Bewerbung für den Wettbewerb „Gesundheit und Wissenschaft“ - Themenfeld: Klinische Forschung - Impferfolg bei Nierentransplantierten

Grundinformationen

Kurzbeschreibung Ihres Tätigkeitsbereiches

Ich leite eine Arbeitsgruppe, die sich mit der zellulären und humoralen Immunantwort bei Transplantierten, also mit der Messung der Funktion ihres Immunsystems beschäftigt. Dabei interessiert mich besonders, wie gut die transplantierten Patienten auf Impfungen ansprechen und ob es nach Impfung möglicherweise zu unerwünschten Wirkungen wie Abstoßungsreaktionen kommt.

Ansprechpartner:in (inkl. Kontaktdaten), kurzer Lebenslauf (s. Anlage)

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Allgemeinverständlich formulierte Zusammenfassung ihrer Arbeit/ Forschungsprojekt und Miteinsendung Ihrer Publikationen (s. Anlage)

Bei Organtransplantierten sind Infektionen und Abstoßungsreaktionen die beiden zentralen Probleme. Impfungen sind die wirksamste Methode, um Infektionen zu verhindern. Allerdings steht nach wie vor die Frage im Raum, wie wirksam Impfungen bei Transplantierten sind und ob durch sie möglicherweise Abstoßungsreaktionen in Gang gesetzt werden könnten. Beide Themen sind ganz entscheidend für die gute Versorgung dieser vulnerablen Patientengruppe und werden in den genannten Arbeiten behandelt. Bei Nierentransplantierten wurden im Labor diagnostische Teste etabliert, um die Impfantwort möglichst sensitiv und umfassend zu messen. Wir konnten zeigen, dass trotz der immunsuppressiven Behandlung, die nach Nierentransplantation notwendig ist, die Patienten eine Impfantwort aufbauen konnten und dass es zu keiner Steigerung von Abstoßungsreaktionen kam. Diese Erkenntnisse haben unmittelbare Konsequenzen für die Therapie, da sie die Impfempfehlung der Ständigen Impfkommission auch für Nierentransplantierte uneingeschränkt unterstützen.

Die vorgelegten Originalarbeiten, die in der Anlage als PDF Dokumente beigefügt sind, beschäftigen sich mit dem Impferfolg bei Nierentransplantierten. Gegen folgende Erreger wurde geimpft:

- *Streptococcus pneumoniae* (führt u.a. zu Lungenentzündungen) (#1-4)
- SARS-CoV-2 (Severe acute respiratory syndrome coronavirus type 2, „Corona“) (#5)
- Varicella-Zoster-Virus (führt u.a. zu Windpocken und Gürtelrose) (#6)

Bewerbung für den Wettbewerb „Gesundheit und Wissenschaft“ - Themenfeld: Klinische Forschung - Impferfolg bei Nierentransplantierten

Zusammenfassende Kurzbeschreibung folgender Punkte

Wie beurteilen Sie das Innovationspotential Ihrer Einsendung?

Die vorgelegten Arbeiten sind äußerst innovativ, da die Impfantworten mit neu etablierten Testverfahren besonders sensitiv und umfassend gemessen wurden. Dabei kam u.a. das ELISpot-Verfahren zum Einsatz, mit dem auf Einzelzellniveau die Funktion weißer Blutzellen gemessen werden kann. Es konnte gezeigt werden, dass trotz der Immunsuppression, die bei Transplantierten eine Abstoßung verhindern soll, die Bildung von Antikörpern gegen Infektionserreger und auch die Bekämpfung der Erreger durch weiße Blutzellen noch möglich ist. Ferner konnten Immunantworten auf bestimmte Untergruppen von Bakterien (die unterschiedlich gefährlich sind) oder auf bestimme Bestandteile von Viren (die entscheidend für den Schutz gegen die Erkrankung sind) nachgewiesen werden. Diese neuen experimentellen Ansätze gehen deutlich über das übliche Untersuchungsspektrum von Laboren hinaus.

Wie beurteilen Sie Ihre Einsendung bezüglich der Nachhaltigkeit?

Die neu etablierten Verfahren konnten so weiterentwickelt werden, dass sie jetzt im Rahmen der Patientendiagnostik verwendet werden können. Damit dient die Neuetablierung von Methoden nicht nur einer spezifischen wissenschaftlichen Fragestellung, sondern sie ist nachhaltig (weil diese Methoden jetzt für die Diagnostik zur Verfügung stehen). Wir können also nicht nur für Nierentransplantierte, sondern auch für Empfänger anderer Organe und für weitere immunsupprimierte Patienten die neuen Teste anbieten.

Leistet Ihre Einsendung einen Beitrag zu einer nachhaltigen Verbesserung der Gesundheit und Lebensqualität?

Ja, die Gesundheit und Lebensqualität einer besonders vulnerablen Patientengruppe kann aufgrund der Ergebnisse der vorgelegten Studien nachhaltig verbessert werden. Es ist davon auszugehen, dass das Impfen der Nierentransplantierten – das aufgrund unserer Daten als wirkungsvoll und sicher erscheint - zu einer Verminderung von Infektionen führt.

Besitzt Ihr Beitrag Standortrelevanz (z.B. für Essen)?

Die vorgestellten Projekte waren nur an einem Standort wie Essen möglich, an dem in der Universitätsmedizin Essen (UME) Experten im Bereich der klinischen Transplantationsmedizin, der Infektiologie, der Nephrologie, der Virologie und der Transplantationsdiagnostik gemeinsam arbeiten. Wir besitzen hier in Essen eines der größten Transplantationszentren in Deutschland, an dem Nieren, Lebern, Herzen und Lungen (also alle vier großen Organe) transplantiert werden können. Nur so konnte eine ausreichend große Patientengruppe geimpft und im Labor mit innovativen Methoden getestet werden. Von den Ergebnissen profitieren unsere transplantierten Patienten, die aufgrund der Ergebnisse unserer sechs interdisziplinären Studien die bestmögliche Behandlung erhalten können, die u. a. in der Impfung gegen Pneumokokken, SARS-CoV-2 und Varizella-Zoster-Virus besteht.

Bewerbung für den Wettbewerb „Gesundheit und Wissenschaft“ - Themenfeld: Klinische Forschung - Impferfolg bei Nierentransplantierten

Anlagen

Lebenslauf

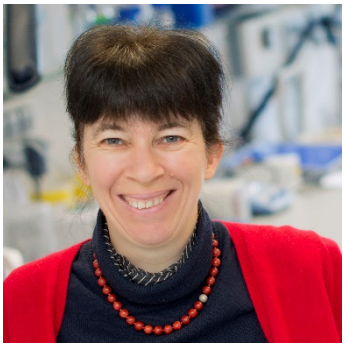
Publikationen

Die vorgelegten Originalarbeiten, die als PDF Dokumente beigefügt sind, beschäftigen sich mit dem Impferfolg bei Nierentransplantierten. Gegen folgende Erreger wurde geimpft:

- *Streptococcus pneumoniae* (#1-4)
- SARS-CoV-2 (#5)
- Varicella-Zoster-Virus (#6)

1. Sequential Vaccination Against *Streptococcus pneumoniae* Appears as Immunologically Safe in Clinically Stable Kidney Transplant Recipients. **Lindemann M**, van de Sand L, Mülling N, Völk KL, Aufderhorst UW, Wilde B, Horn PA, Kribben A, Krawczyk A, Witzke O, Heinemann FM. Vaccines (Basel). 2024 Oct 31;12(11):1244.
2. Antibody responses after sequential vaccination with PCV13 and PPSV23 in kidney transplant recipients. Mülling N, van de Sand L, Völk K, Aufderhorst UW, van der Linden M, Horn PA, Kribben A, Wilde B, Krawczyk A, Witzke O, **Lindemann M**. Infection. 2023 Dec;51(6):1703-1716.
3. Correlation of Fc Receptor Polymorphisms with Pneumococcal Antibodies in Vaccinated Kidney Transplant Recipients. Arnold ML, Heinemann FM, Oesterreich S, Wilde B, Gäckler A, Goldblatt D, Spriewald BM, Horn PA, Witzke O, **Lindemann M**. Vaccines (Basel). 2022 May 5;10(5):725.
4. Establishment of an ELISpot Assay to Detect Cellular Immunity against S. pneumoniae in Vaccinated Kidney Transplant Recipients. Gäckler A, Mülling N, Völk K, Wilde B, Eisenberger U, Rohn H, Horn PA, Witzke O, **Lindemann M.** Vaccines (Basel). 2021 Dec 6;9(12):1438.
5. Cellular and Humoral Immunity against Different SARS-CoV-2 Variants Is Detectable but Reduced in Vaccinated Kidney Transplant Patients. Thümmler L, Gäckler A, Bormann M, Ciesek S, Widera M, Rohn H, Fisenkci N, Otte M, Alt M, Dittmer U, Horn PA, Witzke O, Krawczyk A, **Lindemann M**. Vaccines (Basel). 2022 Aug 18;10(8):1348.
6. Prospective, Longitudinal Study on Specific Cellular Immune Responses after Vaccination with an Adjuvanted, Recombinant Zoster Vaccine in Kidney Transplant Recipients. **Lindemann M**, Baumann C, Wilde B, Gäckler A, Meller L, Horn PA, Krawczyk A, Witzke O. Vaccines (Basel). 2022 May 26;10(6):844.

Lebenslauf/Prof. Dr. med. Monika Lindemann



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Dissertation

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Ärztliche Tätigkeit

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01.10.1994-31.12.1995	AiP in der Abteilung für Endokrinologie und Nieren- und Hochdruckkrankheiten der Medizinischen Klinik am Universitätsklinikum Essen
01.01.1996-31.12.2008	Wissenschaftliche Mitarbeiterin im Institut für Immunologie am Universitätsklinikum Essen (Vertreterin des Institutsdirektors bei der Befundung)
15.03.1996-13.02.2001	Rotationen zu Prof. Roggendorf ins hiesige Institut für Virologie für 3 1/2 Jahre, zu Prof. Ansorg ins hiesige Institut für Medizinische Mikrobiologie für 1/2 Jahr sowie zu Prof. Bertrams ins Zentrum für Labormedizin am Elisabeth-Krankenhaus Essen für 1/2 Jahr
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seit 16.09.2005	Mitglied der Weiterbildungskommission der Deutschen Gesellschaft für Immungenetik (DGI)
22.06.2006	Habilitation für das Fach Immunologie an der Universität Duisburg-Essen (Thema: Charakterisierung von Einflussfaktoren der anti-mikrobiellen zellulären in-vitro Immunantwort bei gesunden und immundefizienten Probanden)
12.10.2006	Anerkennung als Fachimmungenetikerin (DGI)
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12.11.2013	European Specialisation in Histocompatibility and Immunogenetics (ESH) Honorary Diploma (European Federation for Immunogenetics, EFI)
06.09.2014-05.09.2024	EFI Codirector
seit 11.05.2018	Mitglied des Educational Committees (EFI)
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01.07.2020	Erwerb der Zusatzbezeichnung Immunologie (Ärztekammer Nordrhein)
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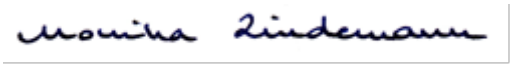
Essen, 21.05.2025 

vaccines

MDPI

Article

Sequential Vaccination Against *Streptococcus pneumoniae* Appears as Immunologically Safe in Clinically Stable Kidney Transplant Recipients

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Abstract: Background: Vaccination against *Streptococcus pneumoniae* is advised for transplant recipients to reduce morbidity and mortality associated with invasive pneumococcal disease. However, data on alloantibodies after sequential vaccination (with a pneumococcal conjugate vaccine followed by a polysaccharide vaccine) are still lacking. **Methods:** In the current study, we determined HLA class I and II and major histocompatibility class I-related chain A (MICA) antibodies in 41 clinically stable kidney transplant recipients. These antibodies were measured prior to and post sequential pneumococcal vaccination over a period of 12 months. Alloantibodies were measured by Luminex bead-based assays, and pneumococcal IgG antibodies were measured by ELISA. **Results:** Over a 12-month period, the sequential analysis revealed no significant change in alloantibodies. One patient developed de novo donor-specific antibodies (DSA) 1.5 months after the first vaccination, with mean fluorescence intensities of up to 2300. These DSA became undetectable in the follow-up, and the patient showed no signs of allograft rejection. Another patient experienced a biopsy-proven borderline rejection 7 months after the first vaccination but did not develop de novo DSA. Both maintained stable kidney function. As expected, the pneumococcal antibodies increased significantly after vaccination ($p < 0.0001$). **Conclusions:** Given the overall risk of alloimmune responses in transplant recipients, we would not attribute the two noticeable patient courses to vaccination. Thus, we consider sequential vaccination immunologically safe.

Keywords: *Streptococcus pneumoniae;* conjugate and polysaccharide vaccines; sequential vaccination; kidney transplant patients; alloresponse; HLA antibodies

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1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a Gram-positive bacterium that forms pairs (diplococcus) and frequently colonizes the human nasopharynx [1]. Outside this region, it can cause otitis media, sinusitis, lobar pneumonia, or meningitis. It is the leading

cause of community-acquired pneumonia globally [2]. In addition to local infection, *S. pneumoniae* can lead to invasive pneumococcal diseases (IPD), which carry an approximately 10% fatality rate [1,3,4]. Data by the Centers for Disease Control and Prevention (CDC) indicate that the rate of IPD in organ transplant recipients is 25 times higher than in the general population [3,4]. To lower the risk of IPD, immunocompromised individuals should be vaccinated against *S. pneumoniae* [5,6].

S. pneumoniae contains an estimated 500 proteins on its surface [7]. The exterior of the bacterium is covered by a polysaccharide capsule and serotypes are determined by the structure of the capsule [7]. The capsular polysaccharides are considered as the main virulence factors of *S. pneumoniae* [8]. Antibodies against these polysaccharides protect against invasive infection [8]. The capsular polysaccharides are nearly always negatively charged and thereby avoid the entrapment of pneumococci in the nasal mucus [9]. They help the bacteria to survive desiccation for many days. Because the polysaccharides cover deeper bacterial surface structures, they inhibit the binding of immunoglobulins, complement components, and C-reactive protein. As described in detail in a recent Nature Review [9], *S. pneumoniae* expresses a plethora of factors that mediate immune evasion. Key mechanisms and structures that enable *S. pneumoniae* colonization also comprise several adhesion molecules, the blockade of IgA1 and the interaction with the complement system, mucus degradation, the binding of ions necessary for immune function, the impairment of neutrophil activity, and the pro-inflammatory effects of concomitant viral infections and toxins such as pneumolysin. The current vaccines against pneumococci target capsular polysaccharides.

The sequential administration of a pneumococcal conjugate vaccine followed by a polysaccharide vaccine was recommended by the CDC and the German advisory board for vaccination (Ständige Impfkommission, STIKO) for immunocompromised individuals such as transplant recipients [5,6,10]. Conjugate vaccines, which are covalently conjugated to carrier proteins, act T cell dependently and promote B cell differentiation into memory B cells or antibody-producing plasma cells. In conjugate vaccines, the capsular polysaccharides are conjugated with the highly immunogenic cross-reactive material 197 (CRM197), a non-toxic diphtheria toxoid protein. CRM197-specific type 2 helper T cells (Th2) interact with B cells that have bound and internalized the polysaccharide-CRM197 complex via polysaccharide-specific IgM, and subsequently present the processed CRM197 protein together with HLA class II to effector T cells [11]. This type of adaptive immune response is characterized by a change in antibody isotypes. On the contrary, polysaccharide vaccines contain a group of T-cell-independent, polyvalent antigens, that is, they can activate B cells without T cell help by crosslinking numerous B cell receptor molecules [12]. There are currently few clinical studies that demonstrate an increase in pneumococcal antibodies after sequential pneumococcal vaccination in solid organ transplant recipients [13,14].

The question of whether immune activation after vaccination may induce allograft rejection has been a matter of debate for several years [15–20]. As demonstrated by Locke et al. [12], proinflammatory conditions, including infection, minor surgical procedures, traumatic injury, and major medical events (e.g., myocardial infarction) may increase alloantibodies. However, data on alloresponses after sequential pneumococcal vaccination are still lacking in transplant recipients.

The objective of the present study was to sensitively monitor (subclinical) alloresponses prior to and post sequential vaccination against pneumococci in patients who have undergone kidney transplantation. The vaccination regimen comprised a 13-valent conjugated vaccine (PCV13), followed by a 23-valent polysaccharide vaccine (PPSV23). The highly sensitive Luminex™-technology-based bead assay was used to measure antibodies against human leukocyte antigen (HLA) class I and class II and against major histocompatibility class I-related chain A (MICA). In parallel, we evaluated pneumococcal IgG antibodies, monitored the occurrence of antibodies against donor-specific antigens (DSA), and assessed the incidence of allograft rejection. We also

monitored kidney function. Finally, we tested whether alloantibodies, pneumococcal antibodies, or kidney function differed between females and males.

2. Materials and Methods

2.1. Patients

A total of 41 clinically stable kidney transplant recipients were included in this cross-sectional, single-center study (Table 1). The median age of the cohort was 59 years (range 33–77 years), with 10 female and 31 male participants. The patients were vaccinated in a sequential manner against *S. pneumoniae*, with an interval of 6 months between both doses. The median interval between the (last) kidney transplantation and the first vaccination was 38 months (ranging from 3 months to 338 months, i.e., 28 years). In order to be included in the study, participants had to meet the following criteria: stable allograft function (defined as a change in serum creatinine concentration below 15% within one month prior to vaccination), an interval of at least three months since kidney transplantation, and the absence of clinical infection, allograft rejection, and pregnancy. Patients who had previously been vaccinated against pneumococci were excluded from the study. The patients were only vaccinated against pneumococci and did not receive a combined vaccination. However, vaccinations against other pathogens were not explicitly excluded during the study period of 12 months. Overall, the patients had a similar level of immune suppression. Blood samples were collected immediately prior to the two vaccinations, one month after the first, and one and six months after the second vaccination (months 7 and 12 after the first vaccination) (Figure 1). Thus, we sequentially tested five blood samples per patient. This study was approved by the institutional review board of the University Hospital Essen (14-5858-BO), and written informed consent was obtained from all participants. It was conducted in accordance with the Declarations of Helsinki and Istanbul and its subsequent amendments.

Table 1. Characteristics of 41 kidney transplant recipients vaccinated sequentially against *S. pneumoniae*.

Parameter	Median (Range) or Number (No.)
Median age (range), years [1]	59 (33–77)
Patient sex (female/male)	10/31
Median interval TX-vaccination (range), months	38 (3–338)
Mean eGFR (range), mL/min/1.73 m^2	
Pre-vaccination	48 (17–109)
Month 1 post-vaccination	48 (15–109)
Month 6 post-vaccination	50 (17–106)
Month 7 post-vaccination	48 (15–105)
Month 12 post-vaccination	47 (16–102)
Immunosuppression, no. [1]	
Cyclosporine A	5
Tacrolimus	30
Mycofenolate mofetil	27
mTOR inhibitors	9
Corticosteroids	38
Kidney transplantation, no.	
1st	37
2nd	4

[1] At the time of the first blood collection; eGFR-estimated glomerular filtration rate; mTOR-mammalian target of rapamycin.

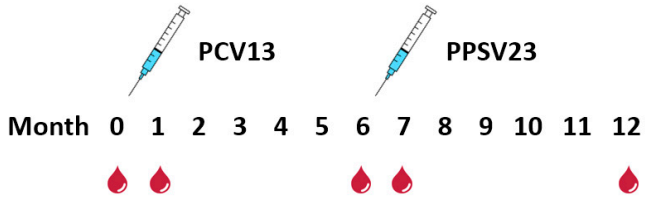


Figure 1. Study design. Sequential pneumococcal vaccination and blood collection in 41 kidney transplant recipients. The blood droplets indicate the time points of blood collection. The first vaccination was performed with a 13-valent conjugated vaccine (PCV13), the second with a 23-valent polysaccharide vaccine (PPSV23). Month 0 indicates baseline (pre-vaccination) and months 1–12 follow-up after the first vaccination. For example, month 7 means 7 months after the first vaccination and 1 month after the second vaccination.

2.2. Vaccines and Vaccination

The first pneumococcal vaccination was performed with 0.5 mL of the 13-valent conjugate vaccine Prevenar 13® (PCV13, Pfizer, Berlin, Germany) that consists of the serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, and 23F. Each of the serotypes is contained at a concentration of 2.2 µg/dose, with the exception of serotype 6B, which is present at a concentration of 4.4 µg per dose. This vaccine is an aluminum-adsorbed conjugate vaccine, where the polysaccharides are individually conjugated to a nontoxic mutant form of diphtheria toxin (CRM197). As additives, it contains 5 mM succinate buffer containing 0.85% NaCl and 0.02% polysorbate 80, at pH 5.8, as well as aluminum phosphate at a dose of 0.125 mg [21].

The 23-valent polysaccharide vaccine Pneumovax 23® (PPSV23, MSD Sharp & Dohme, Haar, Germany), an inactivated vaccine, was used six months later for the second vaccination. The vaccine is unconjugated and contains the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. The vaccine is formulated in phenol and contains <1 mmol sodium chloride per dose (0.5 mL) [22]. Both vaccines were injected into the deltoid muscle.

2.3. Determination of HLA and MICA Antibodies

All patient samples were tested for IgG antibodies against HLA class I and II and MICA using Luminex™-technology (LABScreen-Mixed Beads, catalog ID LSM12NC26_024_01, One Lambda/Thermo Fisher, Canoga Park, CA, USA) in accordance with the manufacturer's instructions and as previously described in detail [23–27]. If results for HLA class I or II antibodies were positive, the Luminex™ Single Antigen Beads (SAB) assay, provided by the same manufacturer, was also employed to ascertain whether the HLA antibodies were donor-specific (DSA) (catalog IDs LS1A04NC26_014_00 (LABScreen Single Antigen Beads for HLA class I) and LS2A01NC26_016_01 (LABScreen Single Antigen Beads for HLA class II)) [24–27].

The Luminex™ assays facilitate the identification of antibodies against antigens that are coupled to fluorescently labeled polystyrene microbeads. As compared to the classical ELISA technique, the Luminex™ method is a multiplex assay that can analyze multiple analytes simultaneously in a single reaction, e.g., antibodies against different HLA molecules. These antibodies bind to the surface of color-coded microbeads using a blend of different fluorescent intensities of two dyes. The commercially available Luminex HLA antibody detection assays provide a higher throughput of samples, the capability of automation, and higher sensitivity as compared to the ELISA. The LABScreen-Mixed Beads Assay comprises twelve beads specific for HLA class I antibodies, five for HLA class II, and two for MICA, respectively. Reactions against each bead were scored as 1 (negative), 4 (undetermined), or 8 (positive). Individual reactions for HLA class I, HLA class II, or MICA were then summed up; yielding antibody score values of 12–96, 5–40, or 2–16,

respectively, as described previously [23,27]. We performed two evaluations with two different cutoffs: A normalized background ratio above 3.0 or above 4.5 was defined as positive, and a range between 2.0 and 3.0 or 3.0 and 4.5, respectively, was defined as undetermined. Finally, a ratio below 2.0 or 3.0 was defined as negative. The first cutoff was used in previous studies of our group [23,27], and the cutoff of 4.5 is the currently applied cutoff for routine samples in our laboratory. Moreover, we evaluated the cumulative mean fluorescence intensities (MFI), where we also summed up responses for antibodies against HLA class I, HLA class II, and MICA, respectively.

2.4. Determination of Antibodies Against Pneumococci

The presence of antibodies against *S. pneumoniae* was determined by an ELISA which detects IgG antibodies against 23 pneumococcal serotypes (VaccZyme™, The Binding Site, Schwetzingen, Germany). The assay was conducted in accordance with the instructions provided by the manufacturer.

2.5. Statistical Analysis

Data were analyzed using GraphPad Prism 8.4.2.679 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics version 25 (Armonk, New York, NY, USA) software. The time course of antibodies was analyzed by 1-way ANOVA, considering a series of five paired samples per patient. Correlation between numerical variables was analyzed by Spearman test. Data in females and males were compared by Mann–Whitney *U* test. Two-sided p-values < 0.05 were considered significant.

3. Results

3.1. Kinetics of Antibody Responses

In 41 kidney transplant recipients, antibodies against HLA class I and II and MICA were determined sequentially, from month 0 (prior to vaccination) until month 12 post-vaccination (Figure 2a). Neither after the first vaccination with the PCV13 (follow-up at month 1) nor after the second vaccination with PPSV23 (at month 7) did we observe a change in HLA or MICA antibodies. In detail, the cumulative antibody score (mean values at months 0, 1, 6, 7, and 12) remained at a similar level for HLA class I (37, 34, 39, 32, and 30), HLA class II (18, 20, 19, 17, and 17) and MICA (7, 6, 7, 6, and 6). The mean cumulative MFI value showed a similar pattern, and no increase was observed after vaccination (Figure 2b).

In contrast, IgG antibodies against pneumococci showed a 1.9-fold increase after the first vaccination ($p < 0.01$ at month 1) and a 3.2-fold increase after the second vaccination ($p < 0.0001$ at month 7), as compared to month 0 (Figure 2c). The antibody concentration increased from a geometric mean of 36 mg/L at month 0 to 61 mg/L at month 1 and month 6 and then further increased to 107 and 97 mg/L at months 7 and 12, respectively.

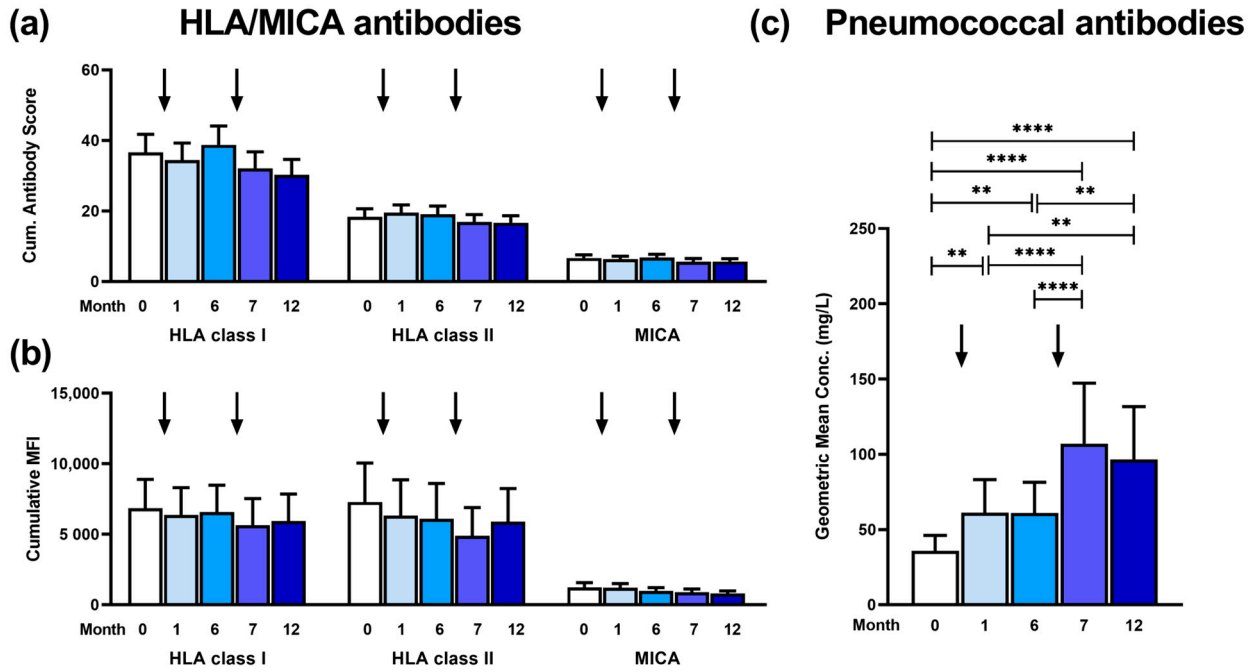
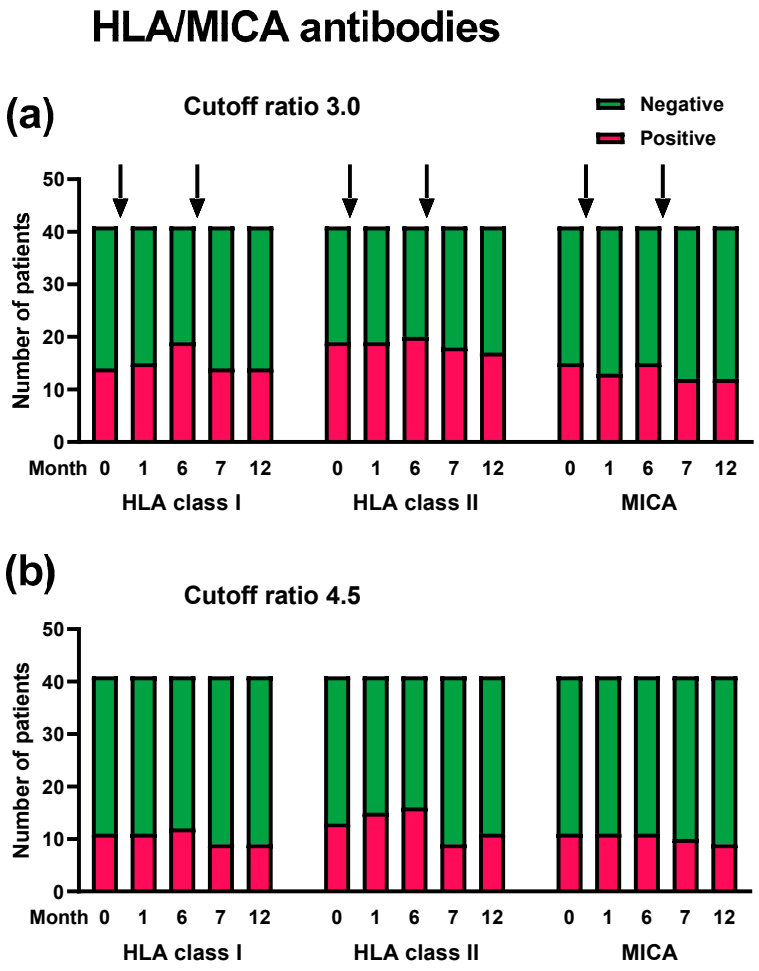


Figure 2. Time-course of antibodies determined in 41 kidney transplant recipients prior to vaccination (month 0, M0) and after vaccination against pneumococci (months 1 to 12, M1–M12). The first vaccination was performed at month 0 (directly after blood collection) with a 13-valent pneumococcal conjugate vaccine and the second vaccination at month 6 with a 23-valent pneumococcal polysaccharide vaccine. The time points of vaccination are indicated by arrows. The left panels show antibodies against human leukocyte antigen (HLA) class I and II and major histocompatibility class I-related chain A (MICA), which were expressed either as cumulative (cum.) antibody scores as detailed in the Methods section (**a**) or as cumulative mean fluorescence intensity (MFI) (**b**). Panel (**c**) indicates IgG antibodies against *S. pneumoniae*. Antibodies against HLA and MICA are presented as mean and standard error of the mean (SEM), pneumococcal antibodies as geometric mean and 95% confidence interval. To compare the results at the various time points, we used a 1-way ANOVA, the Friedman test with Dunn's multiple comparisons test (** $p < 0.01$; **** $p < 0.0001$).

3.2. Patterns of Antibodies Prior to and Post Vaccination

Apart from considering the mean antibody responses, we classified antibodies against HLA and MICA as positive or negative, or we followed up the individual courses of 41 kidney transplant recipients (Figure 3). In summary, there was no significant difference in antibody responses between the five time points. The number of patients with positive antibody responses for HLA class I, HLA class II, or MICA did not increase after the first or second vaccinations. This was observed irrespective of the cutoff ratio set to define a positive response, which was either 3.0 or 4.5 (Figure 3a,b). When comparing data prior to vaccination and at month 12, the number of patients with an increase and decrease in the cumulative antibody score was similar. In detail, 9 patients showed an increase, 20 showed constant values, and 12 showed a decrease in HLA class I antibodies. The respective numbers were 14, 16, and 11 for HLA class II or 3, 31, and 7 for MICA antibodies (Figure 3c). In this analysis, an increase was defined as a difference of ≥1 (month 12–month 0).



(c)

Antibody pattern	Score value (n)		
Pre - M12	HLA class I	HLA class II	MICA
↑	9	14	3
=	20	16	31
↓	12	11	7

Figure 3. Patterns of antibodies in 41 kidney transplant recipients prior to vaccination (month 0, M0) and after vaccination against pneumococci (months 1 to 12, M1–M12). The time points of vaccination are indicated by arrows. Panel (**a**,**b**) indicate the number of patients with positive or negative antibody responses against human leukocyte antigen (HLA) class I and II and major histocompatibility class I-related chain A (MICA), setting the cutoff for positive responses at a ratio of 3.0 (**a**) or 4.5 (**b**), respectively. Panel (**c**) shows changes in the cumulative antibody score, determined pre-vaccination (month 0) and at month 12, i.e., after two vaccinations. Red symbols/numbers indicate patients with an increase (the score in month 12 was at least 1 higher than in month 0), black with constant values, and green with a decrease.

3.3. Correlation of HLA and MICA Antibodies with Clinical Outcome and with Patient Characteristics

One out of 41 patients, a 60-year-old female, developed de novo DSA 1.5 months after the first vaccination (anti-HLA-A1: 1600 MFI; anti-HLA-B60: 2300 MFI) but no biopsy-proven rejection within the follow-up period (Table 2, ID1). Thirteen months after the first vaccination, these low MFI values decreased (anti-HLA-A1: 800 MFI; anti-HLA-B60: 2000

MFI), and 25 months after this vaccination, the MFI values further declined (anti-HLA-A1: 550 MFI; anti-HLA-B60: 1000 MFI). This female patient was vaccinated 10 months after her first kidney transplantation and already displayed very high levels of cumulative antibody scores prior to vaccination (HLA class I: 96, HLA class II: 32), which even decreased at some time points after vaccination. The kidney function remained constant throughout the study period (estimated glomerular filtration rate (eGFR) of 61–69 mL/min/1.73 m^2 and serum creatinine concentration of 1.1–1.2 mg/dL)).

In a second patient, a 56-year-old female, a biopsy-proven borderline rejection was detected 7 months after the first vaccination (ID2). However, she did not develop de novo DSA, and her cumulative antibody scores remained at a similar level. The patient already displayed DSA prior to vaccination, which even showed a slightly lower MFI value at month 1 after vaccination (anti-HLA-B44 at months 0 and 1:2200 MFI and 1300 MFI, respectively). At month 16 after vaccination, DSA were no longer detectable. The patient was vaccinated 22 months after her first kidney transplantation. After vaccination, no change in kidney function could be observed. Even at the time of the borderline rejection, the allograft function remained constant (eGFR of 38 mL/min/1.73 m^2 and serum creatinine concentration of 1.6 mg/dL).

In eight further patients (ID3–8), who also received their first allografts, we observed a major increase in the cumulative antibody score for HLA (at least 10) between month 0 and month 12 (three for HLA class I, four for HLA class II, and one for both, marked red in Table 2). However, none of those patients showed DSA or allograft rejection. Three patients constantly displayed HLA class I and II antibodies (but no DSA) prior to and post vaccination (ID3–5), whereas five patients always had negative results for HLA antibodies, as defined by Luminex™ assays (ID6–10).

In these ten patients (ID1–10) with DSA or borderline rejection or a major increase in the cumulative antibody score, mean kidney function did not change significantly over time, as analyzed by time-series analyses (1-way-ANOVA). The mean eGFR values at months 0, 1, 6, 7, and 12 were 55, 51, 56, 52, and 52 mL/min/1.73 m^2. In the remaining 31 patients, the respective eGFR values also remained at a constant level (46, 47, 47, 46, and 45 mL/min/1.73 m^2).

Table 2. Characteristics of ten kidney transplant recipients with donor-specific antibodies against human leukocyte antigens (HLA), with borderline rejection, or with a major increase of the cumulative antibody score for HLA *.

ID	Sex	Age	HLA Ab **		Cum. Ab Score HLA Class I					Cum. Ab Score HLA Class II					eGFR				
			Class I	Class II	0	1	6	7	12	0	1	6	7	12	0	1	6	7	12
1	F	60	−/+	−/−	96	96	96	92	96	32	32	32	11	26	68	61	69	69	63
2	F	53	+/+	−/−	82	74	82	85	88	11	11	22	15	19	36	38	36	38	29
3	M	62	+/+	+/+	36	53	64	24	46	40	40	40	40	40	29	28	31	31	31
4	F	57	+/+	+/+	12	24	12	12	30	11	20	5	5	20	37	NT	NT	36	31
5	M	47	+/+	+/+	96	96	96	96	96	5	26	22	26	22	106	91	106	91	96
6	M	59	−/−	−/−	12	12	92	96	45	5	5	40	40	14	72	NT	81	76	84
7	M	68	−/−	−/−	12	12	12	12	12	29	17	5	25	40	25	25	20	20	18
8	F	76	−/−	−/−	12	12	12	12	12	5	14	25	17	29	76	NT	53	69	67
9	M	50	−/−	−/−	12	21	12	12	12	21	36	15	21	36	40	54	58	43	50
10	M	59	−/−	−/−	33	32	35	45	54	5	5	8	8	15	60	59	56	47	50

* Defined as increase of at least 10 between month 0 and month 12 after vaccination (marked red). ** Human leukocyte antigen antibodies (HLA Ab) as defined by Luminex Mixed Bead/Single Antigen Bead assay prior to/after vaccination against pneumococci. NT-not tested.

As females may be sensitized against HLA due to pregnancy, we addressed the question of whether HLA or MICA antibodies either prior to or post vaccination were dependent on patient sex (Figure 4a). However, females and males did not differ significantly at

any time point in their cumulative antibody score, as determined by the Mann–Whitney *U* test. However, HLA class I antibodies were slightly higher in females at months 0, 1, and 12. IgG antibodies against pneumococci and kidney function (eGFR) also did not differ significantly between both groups (Figure 4b). But males tended to show higher pneumococcal antibody concentrations at all time points, whereas the eGFR was slightly higher in females.

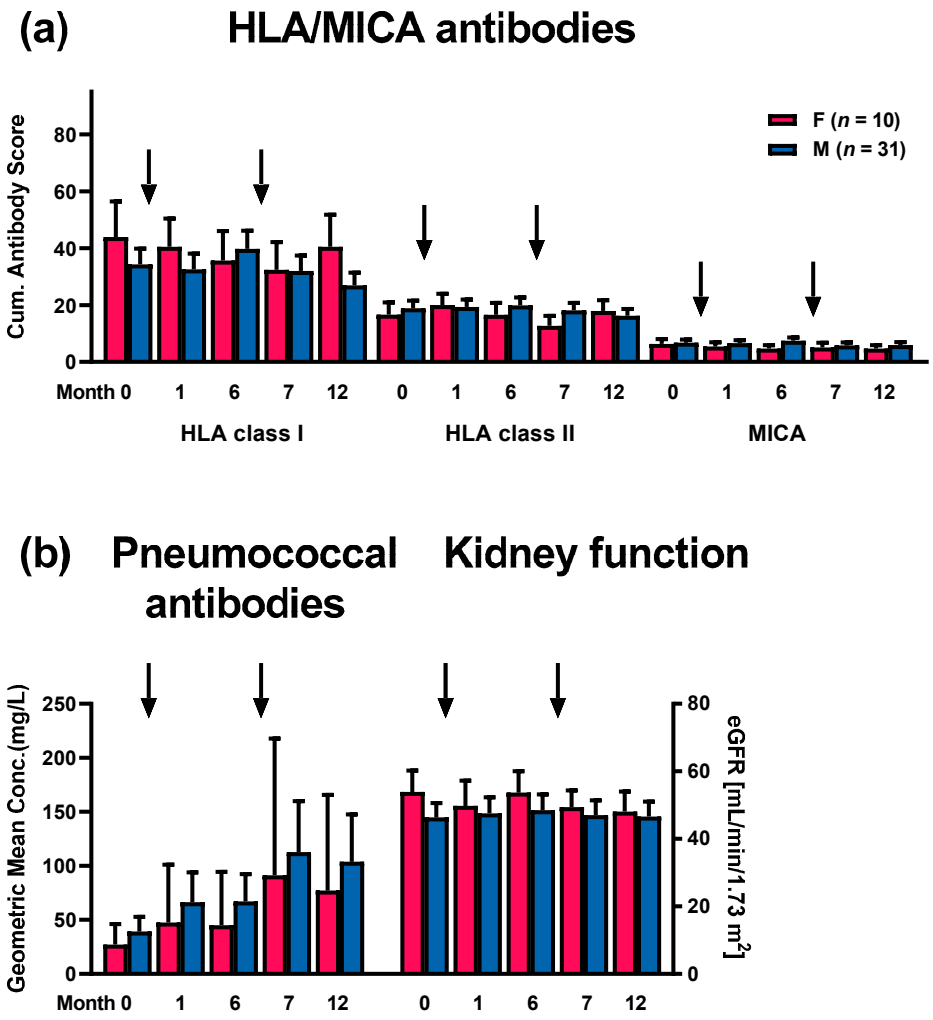


Figure 4. Comparison of antibody patterns in female and male kidney transplant recipients prior to vaccination (month 0, M0) and after vaccination against pneumococci (months 1 to 12, M1–M12). The time points of vaccination are indicated by arrows. Panel (**a**) indicates cumulative antibody scores for antibodies against human leukocyte antigen (HLA) class I and II and major histocompatibility class I-related chain A (MICA); panel (**b**) indicates IgG antibodies against pneumococci and kidney function (estimated glomerular filtration rate, eGFR). Data on alloantibodies and kidney function are presented as mean and standard error of the mean (SEM), and data on pneumococcal antibodies as geometric mean and 95% confidence interval.

Finally, Spearman analysis yielded no significant correlation between HLA or MICA antibodies and age, the interval between transplantation, and vaccination or kidney function (eGFR).

Taken together, the data indicate that sequential vaccination with PCV13 and PPSV23 did not lead to an increase in HLA or MICA antibodies. Even in those patients with clinically noticeable courses or a major increase in cumulative antibody scores, kidney function

remained stable overall. Moreover, we could not find significant differences in the course of antibodies or in kidney function between females and males.

4. Discussion

Taken together, our data argue against the stimulation of alloresponses after sequential pneumococcal vaccination. This result is consistent with a previous publication of our group where kidney transplant recipients received a single vaccination with PPSV23 [23]. Since the vaccine used in the current vaccination regimen, the conjugate vaccine PCV13, induces T-cell-dependent immunity, it could have been possible that the stimulation of T cells leads to cross-reactivity, which may result in alloresponses against the kidney allograft, i.e., the formation of donor-specific antibodies or finally to allograft rejection and the deterioration of kidney function. However, the occurrence of DSA with low MFI values in one patient (which further declined in the follow-up) and the occurrence of one borderline rejection without any change in kidney function may be even less adverse courses than expected within a cohort of 41 patients and a 12-month follow-up. According to registry data, the annual rate of de novo DSA formation after kidney transplantation is 2.5–5% [20,28,29], and that of rejection episodes is 3.6% to 6.4% [20,30]. Moreover, a similar number of patients showed an increase and a decrease in cumulative antibody scores for HLA and MICA within the 12-month follow-up, also arguing against the potential harm of the sequential pneumococcal vaccination.

In 2022, a 20-valent PCV vaccine (PCV20, e.g., Prevenar 20®, Pfizer, Berlin, Germany) was authorized in the European Union [31]. Thereafter, its use was evaluated by the German advisory board for vaccination (STIKO), which in 2024 decided to recommend it for the vaccination of adults with immunodeficiency [32]. Like Prevenar 13®, Prevenar 20® is conjugated to the CRM197 carrier protein, it contains 2.2 µg of each serotype per dose, except for serotype 6B (4.4 µg/dose); it contains succinate and polysorbate 80, and it is adsorbed to aluminum phosphate at 0.125 mg/dose [33]. Due to its composition, it can be assumed that our data on alloimmunity after vaccination with Prevenar 13® can be transferred to Prevenar 20®.

A recent report on 63 kidney transplant recipients, similar to the current study, indicated that two doses of a SARS-CoV-2 mRNA vaccine did not induce anti-HLA antibodies or a significant deterioration of the eGFR [34]. In a further kidney transplant cohort, two out of 100 patients showed de novo DSA after receiving two doses of a SARS-CoV-2 mRNA vaccine [35]. However, in none of the vaccinated patients was an episode of clinically evident acute cellular or antibody-mediated rejection observed, and the authors concluded that SARS-CoV-2 vaccination was not associated with changes in DSA. The findings of the studies on SARS-CoV-2 vaccination were in accordance with the experience with the H1N1 influenza pandemic of 2009. Early reports after the influenza pandemic suggested an alloreactivity to HLA from the H1N1 vaccine (and potent adjuvants have been postulated as causative), but the subsequent review of a large cohort of vaccinated solid organ transplant recipients failed to observe an association [20,27,36]. The review by Mulley et al. [20]—which is the first systematic review to assess de novo DSA and rejection episodes after vaccination in solid organ transplant recipients—included eight prospective controlled clinical studies and did not show an increased rejection risk with vaccination compared with no vaccination (RR 1.12, 95% CI 0.75 to 1.70).

Of note, our own previous data in female kidney transplant recipients indicated that there may be a sex-dependency of HLA antibody formation after a single vaccination with PCV13, and we discussed that females may be more susceptible to the induction of (non-specific) HLA antibodies after vaccination [27]. While 29 males tended to show even lower HLA class I and II antibodies after pneumococcal vaccination, HLA antibodies increased significantly at months 1 and 12 after vaccination in 18 females ($p < 0.05$) [27]. In the current study, we overall observed higher cumulative antibody scores for HLA class I in females than in males. However, this trend could not be observed for HLA class II or for MICA. In line with our previous observation, McCune et al. [35] described that the presence of

DSA before SARS-CoV-2 vaccination was associated with subsequently increased MFI values or with new DSA after vaccination ($p = 0.001$). A similar phenomenon could occur in females after pneumococcal vaccination if they were pre-sensitized due to pregnancies. However, a larger data set is mandatory to definitely address this point.

Why could infection or vaccination induce alloresponses? Memory T cells that are cross-reactive to herpes viruses, especially cytomegalovirus or Epstein–Barr virus, and alloantigens [37,38] as well as inflammation and cytokine production could trigger/enhance alloresponses [39]. Cytomegalovirus infection has been identified as a risk factor for acute and chronic renal allograft rejection [18,40,41]. Immunity to herpes viruses vs. *S. pneumoniae* or the viruses SARS-CoV-2 or influenza is obviously different. While herpes viruses persist for life, the later microorganisms are eliminated after recovery from the infection. Persisting viruses could be harmful to the allograft, whereas microorganisms that are completely eliminated usually appear to be harmless.

The frequency of HLA antibodies we observed by Luminex™ was higher than previously published when setting the cutoff at a ratio of 3.0 (34–46% for HLA class I and 41–49% for HLA class II) and similar to previous studies when setting the cutoff at a ratio of 4.5 (22–27% for HLA class I and 22–39% for HLA class II) [42,43]. Previously, two large studies reported a frequency of HLA antibodies of 30% ($n = 1014$) [42] or of 27% ($n = 4943$) [43]. In our previous study, in which kidney transplant recipients received a single vaccination with PPSV23 [23], the frequencies of HLA and MICA antibodies were overall similar to those found in the current study (using a cutoff ratio of 3.0).

Zhou et al. [44] proposed that "in addition to the adaptive immune response of T and B cells against an alloantigen, MICA is also capable of setting in motion the mechanisms of innate immunity. Co-stimulation by engagement of NK cells might have the effect of potentiating the T and B cell response". It was hypothesized that MICA antigens play a role in human allograft rejection by activating both humoral as well as cellular mechanisms [45]. Data from the 14th International Histocompatibility and Immunogenetics Workshop confirmed this hypothesis and indicated that MICA antibodies were significantly and independently associated with reduced kidney allograft survival in deceased donor grafts, providing strong evidence for the involvement of these antibodies in graft rejection [46]. The frequency of MICA antibodies in the current study of kidney transplant recipients (22–37%, depending on the cutoff) was in the range of a recent publication by Ming et al. (25%) [47].

Immunosuppressive treatment after transplantation—to prevent allograft rejection—leads to impaired cellular and humoral immunity [48]. Corticosteroids induce this impairment of T and B cell immunity by a blockade of transcription factors such as nuclear factor-κB (NF-κB), the calcineurin inhibitors cyclosporine A and tacrolimus by inhibiting the transcription of cytokine genes, especially IL-2, and mycophenolate mofetil by the blockade of DNA synthesis. In a previous study, we showed that four weeks after vaccination, the number of serotypes against which protective pneumococcal antibody concentrations were detectable was significantly lower in 43 kidney-transplant recipients as compared to 75 vaccinated healthy controls, pointing to impaired humoral immunity in kidney transplant recipients [49]. The sum of IgG antibody concentrations (against pneumococci), however, was only non-significantly lower in transplant patients than in the controls (approximately 16% lower). We are not aware of any pneumococcus-specific T cell data analyzing the effect of immunosuppressive treatment. But after vaccination against other viruses, influenza A and B, T cell responses in kidney transplant recipients vs. healthy controls were even more severely suppressed [50]. Influenza-specific proliferative responses were 3-fold lower and those of IFN-γ secretion were 3- to 9-fold lower. In the current study, however, we did not compare a transplant patient cohort with healthy controls.

There are some limitations to this study that need to be considered, such as the lack of a control group or the nature of the study, which is a single-center study. Moreover, it has been discussed that potent adjuvants such as squalene could trigger immune responses that may not be induced after applying vaccines with less potent adjuvants such

as aluminum [27]. It is therefore important to emphasize that the results of the current study relate to the vaccines we used and that the results may be different when using more potent adjuvants. Thus, our findings should be generalized with caution.

In conclusion, the current data on 41 kidney transplant recipients argue against an increase of (subclinical) alloresponses after sequential pneumococcal vaccination. Thus, a vaccination scheme containing PCV13, which induces T-cell-dependent immunity, appears to be immunologically safe in clinically stable kidney transplant recipients.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Essen, Germany (14-5858-BO).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Conflicts of Interest: O.W. has received research grants for clinical studies, speaker's fees, honoraria and travel expenses from Amgen, Alexion, Astellas, Basilea, Biotest, Bristol-Myers Squibb, Correvio, Chiesie, Gilead, Hexal, Janssen, Köhler Chemie, MSD, Novartis, Roche, Pfizer, Sanofi, and TEVA. The remaining authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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RESEARCH

Antibody responses after sequential vaccination with PCV13 and PPSV23 in kidney transplant recipients

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Abstract

Purpose Vaccination against *Streptococcus pneumoniae* is recommended in transplant recipients to reduce the morbidity and mortality from invasive pneumococcal disease. Previous studies indicate that transplant recipients can produce specific antibodies after vaccination with the 13-valent pneumococcal conjugate vaccine Prevenar 13 (PCV13) or the pneumococcal polysaccharide vaccine Pneumovax 23 (PPSV23). National guidelines recommend sequential vaccination with PCV13 followed by PPSV23 in kidney transplant patients. However, there are currently no data on the serological response in kidney transplant recipients, who received a sequential vaccination with PCV13 and PPSV23.

Methods In the current study, we sequentially vaccinated 46 kidney transplant recipients with PCV13 and PPSV23 and determined global and serotype-specific anti-pneumococcal antibody responses in the year following vaccination.

Results Serotype-specific and global anti-pneumococcal antibody concentrations were significantly higher compared to baseline. We observed that serotype-specific antibody responses varied by serotype (between 2.2- and 2.9-fold increase after 12 months). The strongest responses after 12 months were detected against the serotypes 9N (2.9-fold increase) and 14 (2.8-fold increase). Global antibody responses also varied with respect to immunoglobulin class. IgG2 revealed the highest increase (2.7-fold), IgM the lowest (1.7-fold). Sequential vaccination with both vaccines achieved higher antibody levels in comparison with a historical cohort studied at our institute, that was vaccinated with PCV13 alone. During the 12-months follow-up period, none of the patients developed pneumococcal-associated pneumonia or vaccination-related allograft rejection.

Conclusion In conclusion, we strongly recommend sequential vaccination over single immunization in kidney transplant recipients.

Keywords *Streptococcus pneumoniae* · Vaccination · Kidney transplantation · Sequential vaccination · Serotype specific immunity · Pneumococcal antigens

Introduction

Immunocompromised patient cohorts such as solid organ transplant recipients are at major risk for infectious complications. These include lower respiratory tract infections, which can lead to severe disease with requirement of hospital treatment or even transfer to the intensive care unit [1]. *Streptococcus pneumoniae* (*S. pneumoniae*) is a capsulated gram-positive bacterium, that frequently colonizes the human nasopharynx, but can also lead to local and systemic diseases [2].

Apart from meningitis, otitis media and sinusitis, it is the most frequently identified bacterial pathogen in pneumonia [3]. Due to the administration of immunosuppressive agents,

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the risk of invasive pneumococcal disease (IPD) is dramatically increased in solid organ recipients. Therefore, vaccination in immunocompromised individuals is recommended to reduce the incidence of IPD [4–6].

The capsule is the main virulence factor of *S. pneumoniae* and consists of different polysaccharides, which form the basis for the classification of pneumococci into over 90 serotypes. Twenty-three of these serotypes are responsible for 80–90% of infections nowadays [7].

Currently, two types of pneumococcal vaccines are licensed and used in routine clinical practice: the pneumococcal polysaccharide vaccines (PPSV) and pneumococcal conjugate vaccines (PCV). PPSVs act as T-cell independent type 2 antigens, inducing IgG responses and poor generation of memory B cells. PCV was developed to enhance immunogenicity by covalent conjugation to carrier proteins. These peptides induce a T helper cell response, which can promote B-cell differentiation into antibody producing plasma cells or memory B cells [8].

Apart from a reduced immunogenicity of vaccines in solid organ transplant recipients, another concern refers to the risk of triggering allograft rejection through stimulation of alloreactive T and B cells, which is of particular interest in PCVs as they are specifically engineered to increase immune activation [8, 9].

In Germany, a sequential administration of the 13-valent pneumococcal conjugate vaccine (PCV13) followed by the 23-valent pneumococcal polysaccharide vaccine (PPSV23) after 6–12 months is recommended for risk groups including solid organ recipients [6]. It is recommended to control humoral vaccination responses in this cohort. However, it is still unclear to what extent serological titers reflect protection against an infection with *S. pneumoniae* [6]. Studies in kidney transplant recipients (KTR), that examined the humoral response after administration of PCV13 revealed increased functional antibody responses after vaccination [8, 10] but could not match the responses in healthy controls [11]. The administration of PPSV23 in kidney KTR also led to a significant increase of antibodies, which was still detectable after a period of 15 months [12, 13].

However, there are currently no data on the serological response in KTR, who received a sequential vaccination with PCV13 and PPSV23. In addition to a measurement of a global pneumococcal antibody response (against 23 serotypes), we also determined specific immune responses to six pneumococcal serotypes. Therefore, this study aims to investigate the serological immunogenicity and safety of the aforementioned vaccination regiment in KTR.

Materials and methods

Study population

A total of 46 kidney transplant recipients were included in this single-center study between 11/2018 and 10/2019. Basic patient information is given in Table 1. Median age was 57 years (range 22–76 years). Thirty-one patients were male, fifteen patients were female. The median interval between the last kidney transplantation and study inclusion was 38 months (range 3–338 months).

The following criteria led to exclusion from the study:

- Interval between kidney transplantation and study inclusion < 3 months

Table 1 Basic patient characteristics

Parameter	Median (Range) or Number (No.)
Median age (range), years[a]	57 (22–76)
Patient sex (male/female)	31/15
Median interval after kidney transplantation (range), months	38 (3–338)
Median serum creatinine (range), mg/dl	
Pre vaccination	1.58 (0.87–3.55)
Month 6 post vacc	1.55 (0.56–3.65)
Month 12 post vacc	1.57 (0.86–3.87)
Immunosuppression, no. (%)[a]	
Cyclosporine A	6 (13%)
Tacrolimus	35 (76%)
Mycophenolic acid	33 (72%)
mTOR inhibitors	7 (15%)
Corticosteroids	41 (89%)
Belatacept	3 (7%)
Kidney transplantation, no. (%)	
First	42 (91%)
Second	4 (9%)
Comorbidities, no. (%)	
Diabetes mellitus	11 (24%)
Hypertension	31 (67%)
Coronary heart disease	10 (22%)
Co-medication, no. (%)[a]	
Diuretics	18 (39%)
ACE inhibitors/AT1 receptor antagonists	24 (52%)
Calcium chanel blockers	24 (52%)
Beta blockers	29 (63%)
Statins	28 (61%)
Oral anticoagulation	5 (11%)
Insulin	7 (17%)

[a]At the time of first blood sampling; mTOR = mammalian target of rapamycin

- eGFR < 15 ml/min/1.73 m^2
- Acute deterioration of allograft function (related to the definition of acute kidney injury) [14]:
  - o Increase in serum creatinine by ≥ 0.3 mg/dl within 48 h or
  - o Increase in serum creatinine to 1.5 times baseline within the prior 7 days or
  - o Urine volume < 0.5 ml/kg/h for 6 h
- Acute symptomatic bacterial infection with fever > 38.5 degrees
- Allograft rejection within last 6 months
- Pregnancy
- Previous vaccination against *S. pneumoniae* within the last five years

The study design is summarized in Fig. 1. Patients were sequentially vaccinated with a single dose of PCV13 and a single dose of PPSV23 six months later. Blood samples were drawn immediately prior to both vaccinations and at months 1, 7 and 12 post-vaccination, respectively. The patients were followed up for clinical endpoints such as pneumonia or allograft rejection until month 18 after the first vaccination.

This study was approved by the institutional review board of the University Hospital Essen (14–5858-BO), and written informed consent was obtained from all participants. It was carried out in accordance with the Declarations of Helsinki and Istanbul and its subsequent amendments.

Vaccines

The pneumococcal vaccine Prevenar 13 contains polysaccharides of 13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9 V, 14, 18C, 19F, 19A, and 23F), each conjugated to a nontoxic mutant form of diphtheria toxin cross-reactive material 197 (CRM197). It contains 2.2 µg/dose of each of the serotypes, except for serotype 6B at 4.4 µg/dose (0.5 mL) [10].

The pneumococcal vaccine Pneumovax 23 is an unconjugated vaccine that contains 25 µg each of the 23 pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9 V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. Further details on the vaccines have been described in detail previously [15]. Both vaccines were injected into the deltoid muscle [10].

Control groups

We considered two historical control groups, tested for pneumococcal antibodies by the same commercial ELISA as the current study population (VaccZyme™, The Binding Site, Schwetzingen, Germany). The first cohort comprises 47 clinically stable adult kidney transplant recipients, tested at our transplant center after a single vaccination with PCV13 [11, 16]. The second cohort comprises vaccine naïve healthy blood donors described in two previous studies, where reference values were defined for IgG and IgG2 [17] or for IgA and IgM [18]. A third historical control group, healthy controls that received a single vaccination with PCV13 [19], was also considered to compare pneumococcal serotype-specific IgG antibodies.

Measurement of serotype-specific antibody concentrations

Pneumococcal serotype-specific IgG antibody levels were determined for 6 serotypes (2, 3, 6A, 9N, 11A, 14) by a serotype-specific enzyme-linked immunosorbent assay (ELISA). Isotype-specific reference ELISA was performed according to the WHO protocol published at https://www.vaccine.uab.edu/uploads/mdocs/ELISAProtocol(007sp).pdf. In summary, 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were coated with serotype

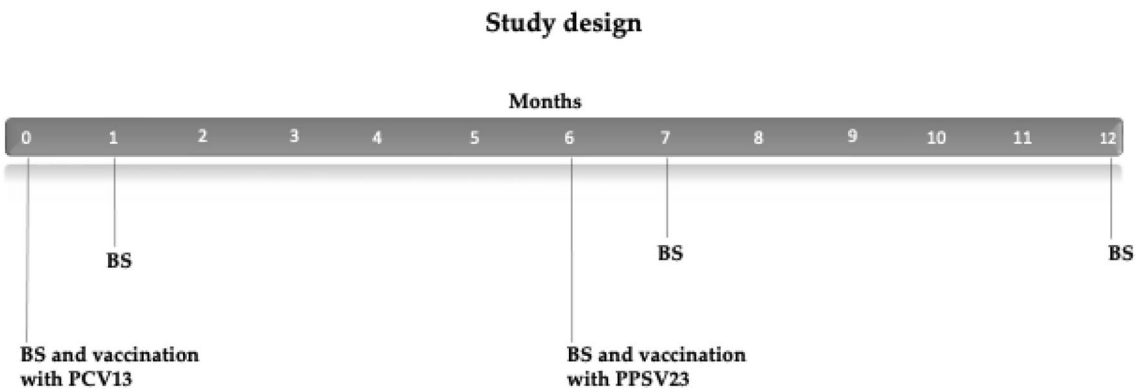


Fig. 1 Study design. All patients sequentially received a single dose of PCV13 and PPSV23. Blood samples (BS) were drawn at the indicated time points

specific pneumococcal polysaccharide (Pn PS) antigen (ATCC, Manassas, VA, USA) and incubated in a humidified chamber at 37 °C for 5 h. Additionally, plates were blocked for 1 h at room temperature with DPBS plus 1% (*w*/*v*) dried nonfat milk powder prior to use. Patient serum samples and standard reference serum (007sp) were pre-incubated with cell-wall polysaccharide (CWPS) and pneumococcal type 22F capsular polysaccharide. Quality control serum was kindly provided by Mustafa Akkoyunlu (Pneumococcal Reference Laboratory, Birmingham, AL, USA). Control and test sera were added to the plate in duplicate wells and incubated at room temperature. The antibodies bound to the plates were detected by an anti-Human IgG (γ-chain specific) F(ab')2-Fragment-Peroxidase for all 4 IgG subclasses (A2290; Sigma-Aldrich, St. Louis, MO, USA). Subsequently TMB ELISA substrate (Thermo Fisher Scientific, Cleveland, OH, USA) reaction was stopped by addition of a sulphonic acid stop solution. Optical density was measured at 450 nm and 690 nm (reference) using Tristar 3 multimode ELISA plate reader (Berthold Technologies, Bad Wildbad, Germany). Serum antibody concentrations were calculated with GraphPad Prism 8.4.2.679 (GraphPad Prism Software, San Diego, CA, USA) by a log linear regression analysis. The lower limit of detection of this assay is approximately 0.01 mg/L.

Determination of global antibody responses against pneumococci

Antibodies against *S. pneumoniae* were determined by four commercial ELISA formats which detect IgG, IgG2, IgA and IgM antibodies against 23 pneumococcal serotypes (VaccZyme™), which are the same serotypes as those included in the PPSV23. This assay, which we called global pneumococcal ELISA, was performed according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using GraphPad Prism or IBM SPSS Statistics version 25 (Armonk, NY, USA). We first checked the data for normal distribution using Shapiro–Wilk test. As various variables did not reveal normal distribution, we applied non-parametric methods to further analyze our data. ELISA responses at different time points were analyzed using the Kruskal–Wallis test, with Dunn´s multiple comparisons test. Continuous variables were compared using the Mann–Whitney *U* test. Correlation analyses of numerical variables were performed using the Spearman test (two-tailed). The impact of MPA treatment and the interval between transplantation and first vaccination on pneumococcal antibodies was analyzed using multivariate analysis (logistic regression). If not otherwise stated, median values are indicated. Results were considered significant at $p < 0.05$.

Results

Clinical course of the study population

All patients were clinically examined until month 18 after the first vaccination. No hospitalized pneumonia with detection of *S. pneumoniae* occurred. One patient had a bacterial superinfection after pneumonia caused by influenza virus, but no certain bacterial pathogen could be identified. Donor-specific antibodies (DSA) were analyzed in 43 patients during the study period; one patient developed de novo DSA 1.5 months after first vaccination (MFI 1600), but no allograft rejection was detected in this patient. A biopsy proved rejection was detected in another patient (histopathological: borderline rejection (Banff category 3), who did not develop de novo DSA.

Pneumococcal antibodies prior to and post vaccination

We tested 230 sera from 46 patients by the standardized WHO pneumococcal ELISA (Fig. 2). Serotype-specific antibody concentrations were determined for a total of six serotypes present in either PCV13 (serotype 6A), PPSV23 (serotypes 2, 9N, 11A), or both vaccines (serotypes 3, 14). Comparing pre-vaccination and month 12 antibody concentrations, a significant increase was observed for each serotype ($p < 0.0005$). Highest values were measured generally at month 7, 1 month after vaccination with PPSV23. In the course of 6 months, there was no significant (n.s.) decrease of antibody levels after either of the two vaccinations. Geometric mean concentration (GMC) was lowest and increased second lowest for serotype 3. The GMC of serotype 3 antibodies increased from 0.2 mg/L pre-vaccination to 0.5 mg/L at month 1 ($p < 0.0005$) and showed highest values with 0.6 mg/L at month 7 ($p < 0.0001$). Only serotype 2 antibodies showed a smaller percentage increase. At pre-vaccination, the GMC of serotype 2 antibodies was 2.7 mg/L which raised up to 6.0 mg/L at month 7 ($p < 0.0001$). As expected, no significant increase was observed in the first 6 months after vaccination with PCV13 for antibody levels against serotypes 2, 9N and 11A, as these are only present in PPSV23. However, serotype 9N showed the strongest increase (2.9-fold) compared to the initial value (Figs. 2, 3). At month 7 after study initiation (1 month after administration of the second vaccination), antibody concentrations against serotype 9N were constant at 2.3 mg/L ($p < 0.0001$). Serotype 14, which has been vaccinated twice as this serotype is included in both PCV13 and PPSV23, reached the

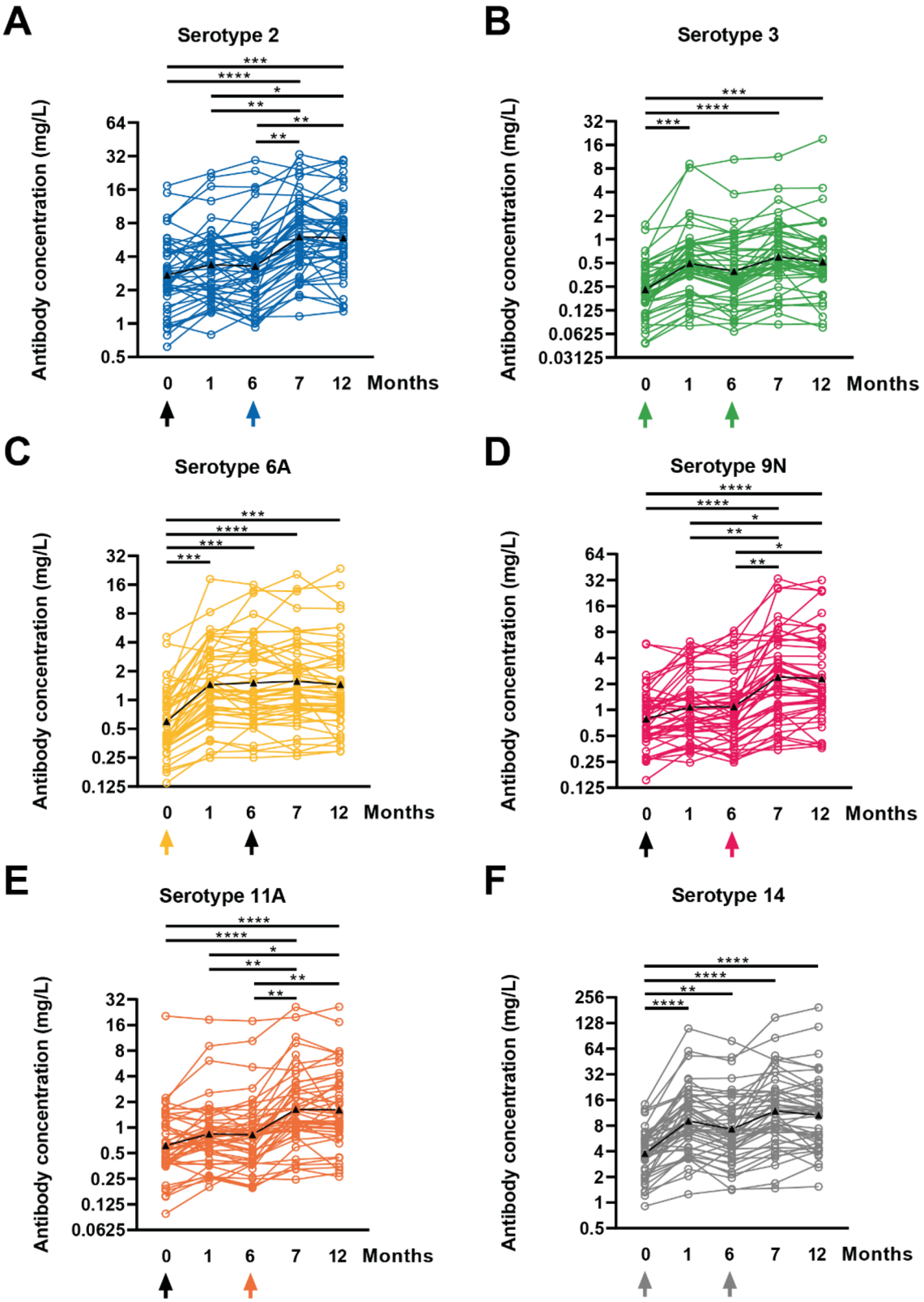


Fig. 2 Individual time courses of serotype-specific pneumococcal antibodies prior to vaccination and after vaccination with PCV13 (at month 0) and with PPSV23 (at month 6). Panels (**A–F**) show serotypes 2, 3, 6A, 9N, 11A and 14. The time of vaccination is indicated by an arrow. Colored arrows denote that the vaccine contains the corresponding serotype. The black line with triangles indicates the geometric mean values. The results are shown on a logarithmic scale (log2). Data in 46 kidney transplant recipients were analyzed by Kruskal–Wallis test, with Dunn´s multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$, ****$p < 0.0001$

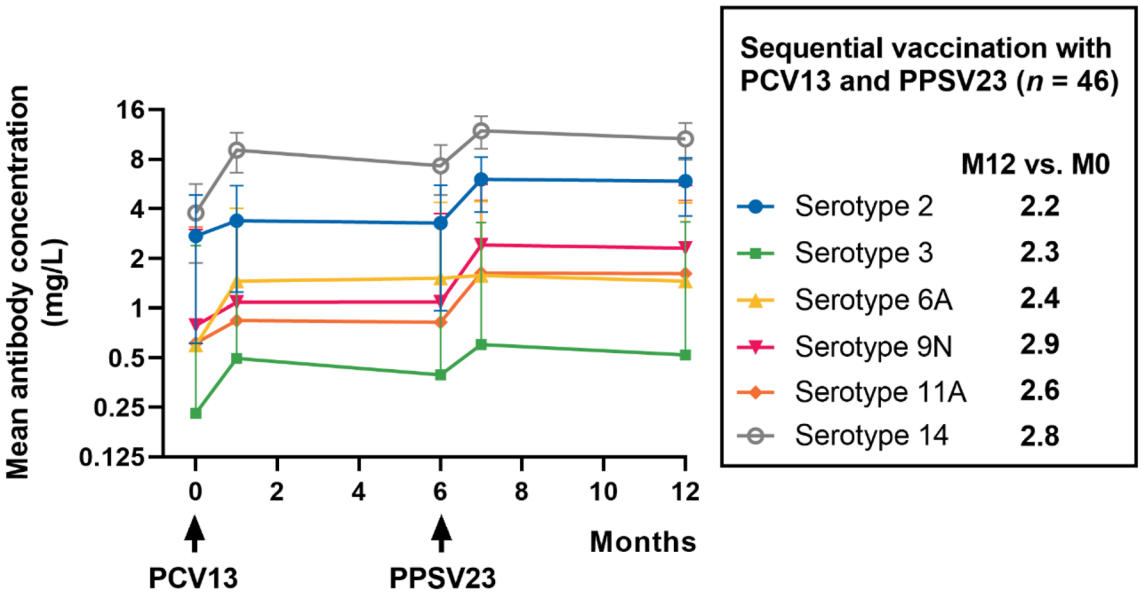


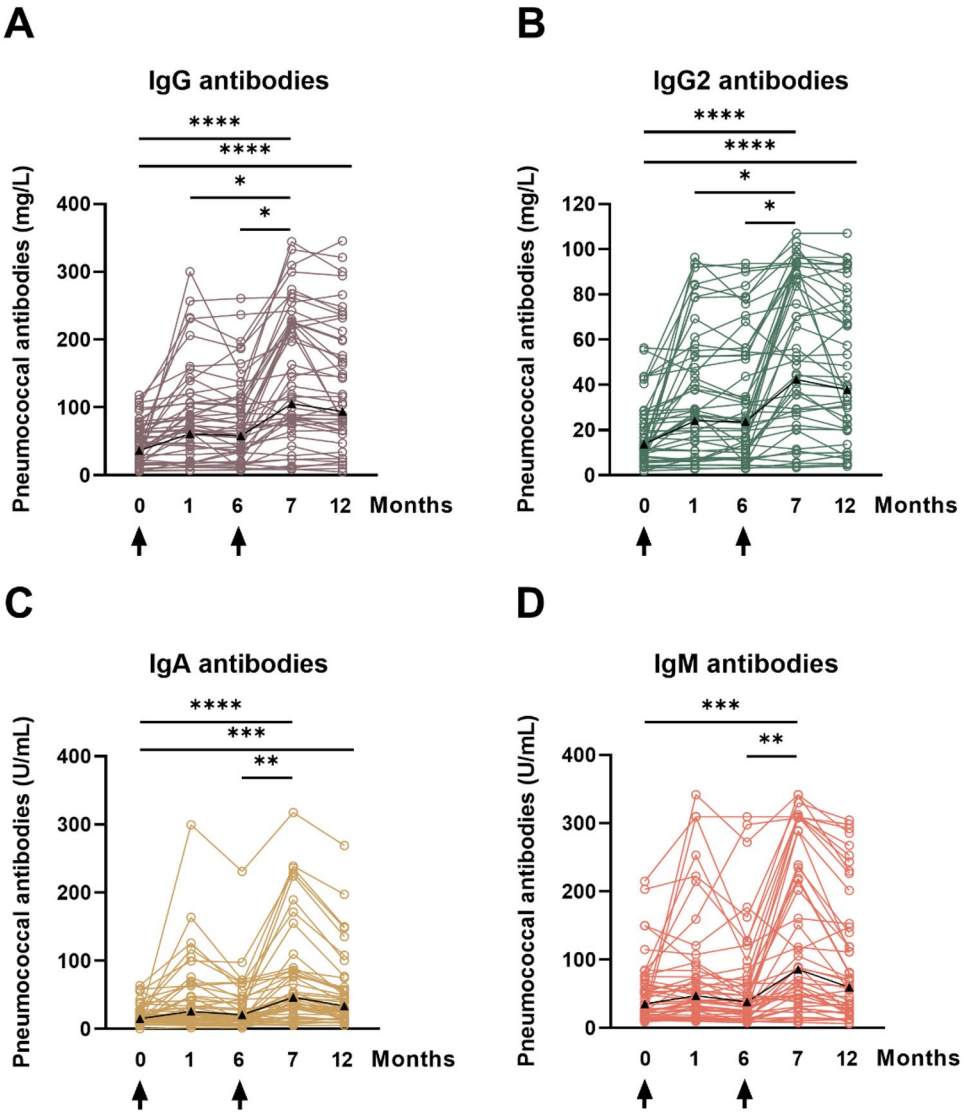
Fig. 3 Concentrations of serotype-specific pneumococcal antibodies in time course of 12 months after first vaccination. Antibodies were determined prior to first vaccination with PCV13 (M0), 1 month after (M1), prior to second vaccination with PPSV23 (M6), at month 7 (M7) and month 12 (M12) thereafter for 46 kidney transplant recipients. Pneumococcal antibodies are given as geometric mean concentration and geometric standard deviation factor. The time of vaccination is indicated by an arrow

highest antibody concentrations. At month 7 it reached a GMC of 11.9 mg/L ($p < 0.0001$). The 2.8-fold increase of antibodies against serotype 14 was the second strongest. A less than twofold increase in concentrations of antibodies against all six serotypes compared to baseline, was observed in only 3 out of all 46 patients. Detailed time courses of antibody concentrations before and after vaccination are presented in Table 2. For the six serotypes, the twofold increase rate of anti-pneumococcal antibodies varied from 54.4% to 71.7% at month 12 (Table 2).

The global IgG, IgG2, IgM and IgA ELISA were performed for 23 serotypes in 230 serum samples. The GMC of IgG antibodies against 23 serotypes was 36.5 mg/L prevaccination, 61.1 mg/L at month 1, 58.1 mg/L at month 6, 105.5 mg/L at month 7 and 94.3 mg/L at month 12 (Figs. 4A, 5A, Table 3). We observed a significant increase ($p < 0.0001$) of IgG antibodies at month 7 and 12, as compared to baseline. Moreover, responses at month 1 and 7 and at month 6 and 7 differed significantly ($p < 0.05$), i.e., antibody levels increased after receiving the second vaccination (with PPSV23). The course of IgG2 and IgG antibody levels was almost identical, however at lower concentrations, as expected (Figs. 4A, B, 5A). IgA and IgM antibody levels also showed a significant increase at month 7, after two vaccinations (Figs. 4C, 4D, 5A). Whereas IgA antibody levels remained significantly increased at month 12, as compared to baseline, the increase of IgM antibody levels was no longer significant at month 12.

Table 2 Serologic responses to sequential pneumococcal vaccination

Serotype	2	3	6A	9N	11A	14
Geometric mean concentration (IQR) mg/L, $n = 46$						
Geometric mean fold increases (IQR), $n = 46$						
Month 0	2.7 (1.9–4.3)	0.2 (0.1–0.3)	0.6 (0.3–0.9)	0.7 (0.4–1.2)	0.6 (0.3–1.0)	3.7 (2.3–5.5)
Month 1	3.3 (1.9–5.2)	0.5 (0.3–0.8)	1.4 (0.7–2.8)	1.0 (0.5–1.7)	0.8 (0.5–1.1)	9.0 (4.4–15.8)
	1.2 (0.8–1.5)	2.1 (1.6–2.6)	2.4 (1.7–3.3)	1.3 (0.9–1.7)	1.3 (0.9–2.0)	2.4 (1.5–3.0)
Month 6	3.2 (1.7–5.1)	0.3 (0.2–0.7)	1.5 (0.7–3.1)	1.0 (0.5–1.9)	0.8 (0.4–1.2)	7.3 (4.4–12.4)
	1.2 (0.8–1.6)	1.7 (1.0–2.3)	2.5 (1.6–3.6)	1.3 (0.9–1.9)	1.3 (0.9–2.1)	1.9 (1.1–2.9)
Month 7	6.0 (3.5–9.9)	0.6 (0.3–1.1)	1.5 (0.8–3.0)	2.4 (1.0–6.2)	1.6 (0.9–2.8)	11.8 (6.6–20.0)
	2.2 (1.4–8.2)	2.6 (1.7–3.9)	2.6 (1.7–3.8)	3.0 (1.7–5.3)	2.6 (1.6–3.9)	3.1 (1.9–4.4)
Month 12	5.8 (3.9–8.6)	0.5 (0.3–0.9)	1.4 (0.7–2.7)	2.3 (1.1–5.5)	1.6 (0.8–3.2)	10.6 (5.1–18.7)
	2.1 (1.4–9.3)	2.2 (1.3–3.8)	2.4 (1.6–3.6)	2.9 (1.6–4.1)	2.6 (1.3–4.3)	2.8 (1.8–3.8)



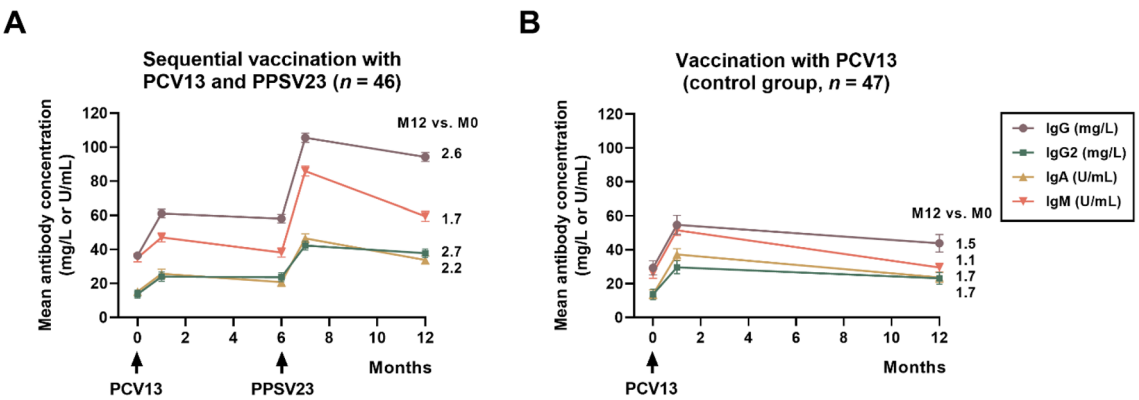


Fig. 4 Individual time courses of pneumococcal antibodies prior to vaccination and after vaccination with PCV13 (at month 0) and with PPSV23 (at month 6). The time of vaccination is indicated by an arrow. Panel (**A**) shows IgG antibodies, panel (**B**) IgG2, panel (**C**) IgA and panel **(D)** IgM, as determined by a commercially available ELISA, measuring antibodies against 23 serotypes (global ELISA). Data in 46 kidney transplant recipients were analyzed by Kruskal–Wallis test, with Dunn´s multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$, ****$p < 0.0001$

Fig. 5 Time courses of pneumococcal antibodies in kidney transplant recipients. Panel (**A**) shows data of the current cohort of 46 patients vaccinated sequentially with PCV13 (at month 0) and with PPSV23 (at month 6). Panel (**B**) displays the course of antibodies in a historical control group of 47 kidney transplant recipients from our transplant center after a single vaccination with PCV13 (at month 0) [11, 16]. Both cohorts were tested for IgG, IgG2, IgA and IgM antibodies by the same commercially available ELISA, measuring antibodies against 23 serotypes (global ELISA). Pneumococcal antibodies are given as geometric mean concentration and geometric standard deviation factor. The time of vaccination is indicated by an arrow

Table 3 Time course of pneumococcal antibody levels in 46 kidney transplant recipients vaccinated with PCV13 at month 0 and with PPSV23 at month 6 and comparison of their antibody concentrations with a healthy reference cohort

Antibody subclass	IgG		IgG2		IgA		IgM	
Time point	GMC	PRC	GMC	PRC	GMC	PRC	GMC	PRC
Month 0	36.5	46	13.8	34	15.2	29	35.1	34
Month 1	61.1	71	24.1	63	25.7	51	47.1	46
Month 6	58.1	66	23.7	61	20.8	49	38.2	34
Month 7	105.5	83	42.3	80	46.5	76	86.0	71
Month 12	94.3	83	37.9	80	33.9	68	59.5	61

GMC geometric mean concentration (given for IgG and IgG2 as mg/L and for IgA and IgM as U/mL), *PRC* percentage comparable to reference cohort

The reference group comprises vaccine naïve healthy blood donors. Their pneumococcal antibodies were used for comparison. Reference values were defined as ≥ 43.8 mg/L for IgG [17], ≥ 20.5 mg/L for IgG2 [17], ≥ 21.0 U/ml for IgA [18] and ≥ 54.0 U/ml for IgM [18]), respectively.

The course of antibodies was compared with an independent control group of kidney transplant recipients, tested at our transplant center after a single vaccination with PCV13 between 2014 and 2015 [11] (Fig. 5). This cohort had a median age of 53 years and the median interval between kidney transplantation and vaccination was 49 months. Data after vaccination with PCV13 were overall very similar in the current cohort and the historical control group. Follow-up data at month 12 indicate that after sequential vaccination geometric mean concentrations of IgG, IgG2, IgA and IgM antibodies were 2.6-fold, 2.7-fold, 2.2-fold, and 1.7-fold higher than prior to vaccination, respectively (Fig. 5A). After a single vaccination with PCV13, we observed 1.5-, 1.7-, 1.7-, and 1.1-fold higher antibodies at month 12 as compared to baseline, respectively (Fig. 5B).

Next, we compared antibody concentrations of our patient cohort with two historical groups of healthy controls. To evaluate global anti-pneumococcal antibody concentrations, we analyzed which fraction of patients showed antibody concentrations comparable to a healthy, vaccine naïve reference group (GMC defined as ≥ 43.8 mg/L for IgG [17], ≥ 20.5 mg/L for IgG2 [17], ≥ 21.0 U/ml for IgA [18] and ≥ 54.0 U/ml for IgM [18]). Prior to vaccination, 29–46% of the patients exceeded the respective threshold, depending on the subclass of pneumococcal antibodies (Table 3). At month 7, when the maximum response was achieved, 83% of patients had IgG antibody levels comparable to those of the reference group, and 80% had comparable IgG2, 76% IgA and 71% IgM antibody concentrations. In summary, kidney transplant recipients display lower baseline-levels of global anti-pneumococcal antibody concentrations across all subclasses compared to healthy controls. However, after sequential vaccination, the majority of kidney transplant recipients show higher antibody concentrations than vaccine naïve healthy controls. The serotype-specific antibody concentrations were compared to another group of healthy individuals who received a single dose of PCV13 [19]. With respect to the tested serotypes, we could compare the values of serotypes 3, 6A and 14. One month after vaccination with PCV13, healthy individuals displayed higher antibody concentrations than our patient cohort (serotype 3: 1.4 vs. 0.5 mg/L; serotype 6A: 7.9 vs. 1.4 mg/L; serotype 14: 12.0 vs. 9.0 mg/L). Twelve months after receiving PCV13, GMC of serotype 6A (only part of PCV13) was still higher in healthy individuals (4.4 mg/L vs. 1.4 mg/L), whereas antibody concentrations of serotypes 3 and 14 (part of PCV13 and PPSV23) were similar or even lower compared to our study population (serotype 3: 0.6 mg/L vs. 0.5 mg/L; serotype 14: 7.7 mg/L vs. 10.6 mg/L).

Spearman correlation analysis of pneumococcal antibody concentrations at various time points and of various subclasses showed the strongest correlation between IgG and IgG2 antibody concentrations, reaching statistical significance ($p < 0.001$) at all time points as indicated by red color (Fig. 6). Overall, IgG and IgG2 antibody levels showed stronger correlation with IgA than with IgM antibody levels. Within each subclass, antibody levels at month 0 correlated significantly with all subsequent time points. Thus, pneumococcal antibody levels prior to vaccination were predictive of antibody levels after vaccination, reaching the highest correlation coefficients for IgM antibody levels.

We also performed correlation analyses between global IgG values and serotype specific antibody concentrations at the respective time-points. With exception of serotype 2 a significant correlation was detectable at most time points after vaccination, reaching the highest correlation coefficients for serotype 9N (Table 4).

Association between clinical patient characteristics and pneumococcal antibody levels

Global IgG, IgG2, IgM and IgA antibody concentrations did not differ significantly between male and female patients at any time-point. There was also no significant correlation between age or allograft function (creatinine, eGFR) and global antibody concentrations. However, Spearman analysis

		IgG 0	IgG 1	IgG 6	IgG 7	IgG 12	IgG2 0	IgG2 1	IgG2 6	IgG2 7	IgG2 12	IgA 0	IgA 1	IgA 6	IgA 7	IgA 12	IgM 0	IgM 1	IgM 6	IgM 7	IgM 12
IgG	0	1	0.60	0.60	0.62	0.66	0.98	0.56	0.54	0.54	0.62	0.32	0.18	0.21	0.13	0.22	0.14	0.08	0.05	0.09	0.09
IgG	1	0.60	1	0.95	0.80	0.76	0.60	0.95	0.91	0.77	0.74	0.11	0.56	0.55	0.47	0.56	0.09	0.43	0.38	0.51	0.47
IgG	6	0.60	0.95	1	0.76	0.76	0.59	0.90	0.94	0.74	0.75	0.11	0.48	0.57	0.39	0.48	0.09	0.41	0.38	0.47	0.44
IgG	7	0.62	0.80	0.76	1	0.95	0.60	0.72	0.69	0.93	0.91	0.07	0.39	0.35	0.51	0.55	0.10	0.35	0.28	0.56	0.47
IgG	12	0.60	0.76	0.76	0.95	1	0.64	0.71	0.71	0.89	0.97	0.11	0.33	0.33	0.47	0.53	0.17	0.35	0.29	0.56	0.49
IgG2	0	0.98	0.60	0.59	0.60	0.64	1	0.59	0.56	0.54	0.62	0.35	0.19	0.21	0.13	0.23	0.19	0.12	0.09	0.11	0.14
IgG2	1	0.56	0.95	0.90	0.72	0.71	0.59	1	0.94	0.75	0.73	0.22	0.60	0.60	0.50	0.60	0.01	0.37	0.32	0.46	0.42
IgG2	6	0.54	0.91	0.94	0.69	0.71	0.56	0.94	1	0.72	0.74	0.23	0.55	0.67	0.47	0.58	0.11	0.41	0.40	0.49	0.48
IgG2	7	0.54	0.77	0.74	0.93	0.89	0.54	0.75	0.72	1	0.91	0.18	0.39	0.41	0.52	0.56	0.16	0.35	0.29	0.54	0.48
IgG2	12	0.62	0.74	0.75	0.91	0.97	0.62	0.73	0.74	0.91	1	0.15	0.30	0.32	0.43	0.51	0.19	0.35	0.27	0.54	0.49
IgA	0	0.32	0.11	0.11	0.07	0.11	0.35	0.22	0.23	0.18	0.15	1	0.48	0.62	0.33	0.40	0.41	0.22	0.21	0.09	0.16
IgA	1	0.18	0.56	0.48	0.39	0.33	0.19	0.60	0.55	0.39	0.30	0.48	1	0.87	0.75	0.78	0.17	0.48	0.39	0.50	0.43
IgA	6	0.21	0.55	0.57	0.35	0.33	0.21	0.60	0.67	0.41	0.32	0.62	0.87	1	0.68	0.74	0.29	0.52	0.50	0.50	0.49
IgA	7	0.13	0.47	0.39	0.51	0.47	0.13	0.50	0.47	0.52	0.43	0.33	0.75	0.68	1	0.96	0.25	0.49	0.43	0.72	0.61
IgA	12	0.22	0.56	0.48	0.55	0.53	0.23	0.60	0.58	0.56	0.51	0.40	0.78	0.74	0.96	1	0.31	0.54	0.49	0.72	0.68
IgM	0	0.14	0.09	0.09	0.10	0.17	0.19	0.06	0.11	0.16	0.19	0.41	0.17	0.29	0.25	0.31	1	0.80	0.79	0.52	0.67
IgM	1	0.08	0.43	0.41	0.35	0.35	0.12	0.37	0.41	0.35	0.35	0.22	0.48	0.52	0.49	0.54	0.80	1	0.95	0.79	0.84
IgM	6	0.05	0.38	0.38	0.28	0.29	0.09	0.32	0.40	0.29	0.27	0.21	0.39	0.50	0.43	0.49	0.79	0.95	1	0.77	0.86
IgM	7	0.09	0.51	0.47	0.56	0.56	0.11	0.46	0.49	0.54	0.54	0.09	0.50	0.50	0.72	0.72	0.52	0.79	0.77	1	0.93
IgM	12	0.09	0.47	0.44	0.47	0.49	0.14	0.42	0.48	0.48	0.49	0.16	0.43	0.49	0.61	0.68	0.67	0.84	0.86	0.93	1

$p < 0.05$ $p < 0.01$ $p < 0.001$

Fig. 6 Spearman correlation analysis of pneumococcal antibodies in 46 kidney transplant recipients vaccinated sequentially with PCV13 (at month 0) and with PPSV23 (at month 6). IgG, IgG2, IgA and IgM antibody concentrations were determined using a commercially available ELISA, measuring antibodies against 23 serotypes (global ELISA). The time points (month 0-month 12) are indicated by numbers (0–12) in the heading. The numbers in the table indicate correlation coefficients, which are color-coded according to the level of significance

Table 4 Spearman correlation analysis of global IgG values and serotype specific IgG concentrations

	IgG 0	IgG 1	IgG 6	IgG 7	IgG 12
Pn PS-2	0.18	0.19	0.16	0.19	0.23
Pn PS-3	0.23	0.39	0.42	0.39	0.18
Pn PS-6A	0.10	0.37	0.39	0.32	0.30
Pn PS-9N	0.31	0.39	0.39	0.47	0.46
Pn PS-11A	0.19	0.27	0.32	0.37	0.33
Pn PS-14	0.19	0.40	0.40	0.29	0.32

Each column shows the correlation coefficient for global IgG values and serotype specific IgG concentrations at the same time point (month 0–12, indicated as 0–12)

$p < 0.05$, $p < 0.01$

indicated a significant positive correlation between global IgG antibody levels and interval between (last) transplantation and first vaccination or subsequent follow-up analyses, with the highest correlation coefficient at month 12 ($r = 0.48$, $p = 0.01$). We also observed a positive correlation between this interval and global IgG2 antibody levels reaching statistical significance at month 7 ($r = 0.40$, $p = 0.006$) and month 12 ($r = 0.42$, $p = 0.003$). Thus, patients vaccinated later after transplantation had higher anti-pneumococcal IgG and IgG2 responses, especially in the long-term follow-up at month 12. However, this observation did not reach statistical significance in the cases of global IgA and IgM antibody levels.

We also analyzed the association between the immunosuppressive regiment and global antibody responses. As the vast majority of patients had a calcineurin inhibitor-based scheme (Table 1), we could not perform an analysis for this treatment, but we compared patients with and without intake of mycophenolic acid (MPA). Patients who did not take MPA had significantly higher global IgG and IgG2 antibody levels at all time-points after vaccination (Fig. 7). There was no significant difference between both groups regarding global IgM and IgA antibody levels. As only the interval between transplantation and vaccination and MPA treatment revealed significant differences in the current study, we included both as independent variables in a logistic regression model to further determine their impact on global IgG and IgG2 antibody levels. The analysis demonstrated that only treatment of MPA proved a significant association with global IgG and IgG2 antibody levels at months 7 and 12.

Discussion

The current study examined the serological immunogenicity and safety of a sequential vaccination with PCV13 and PPSV23 in kidney transplant recipients. We could

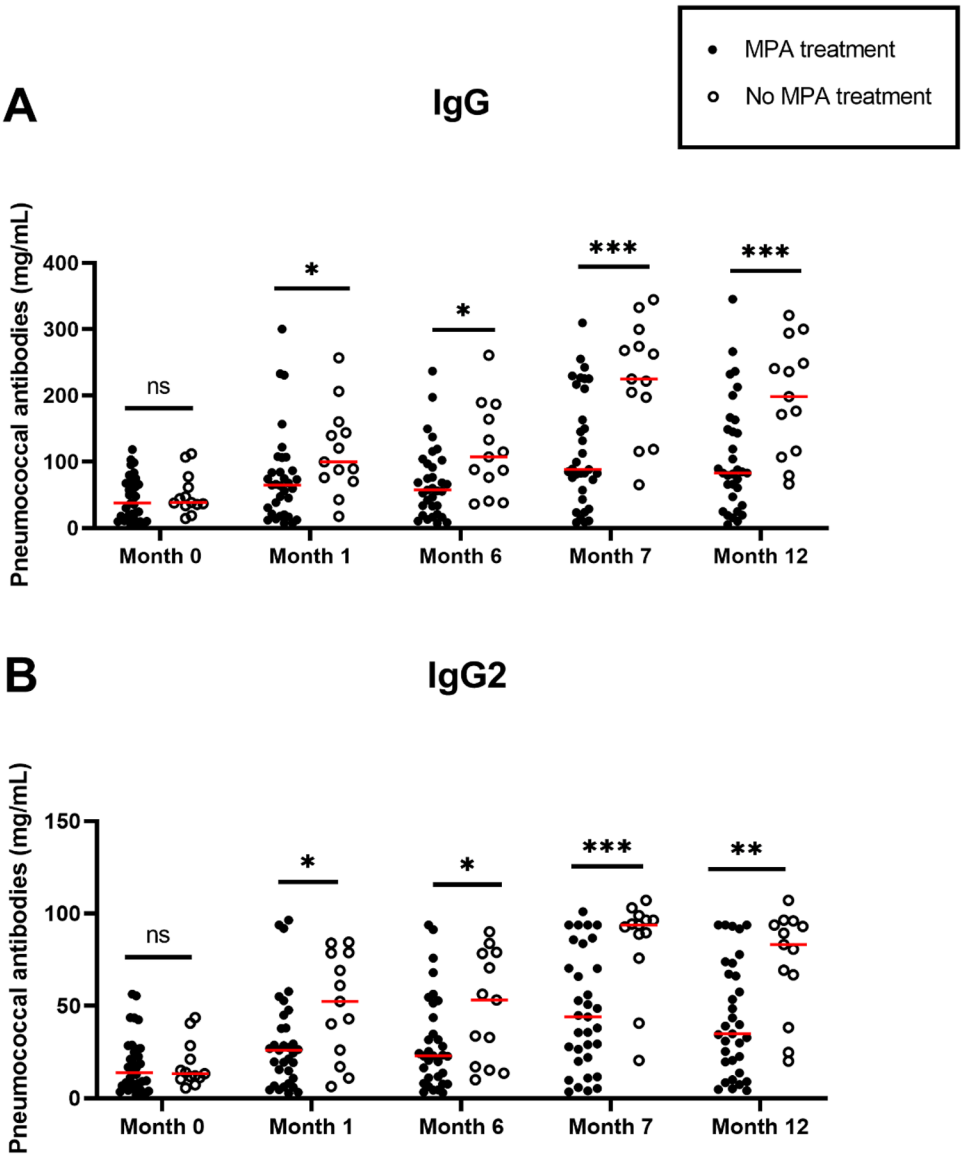


Fig. 7 Comparison of global IgG (**A**) and IgG2 (**B**) antibody responses between patients with (n = 33) and without mycophenolic acid (MPA) treatment (n = 13). Mann–Whitney U test was performed for comparison between groups. After logistic regression analysis, only differences at month 7 and month 12 proved to be significant for both antibody classes. Red lines indicate median values. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$

demonstrate that this vaccination regiment induced a serological response indicated by a significant increase in both, global and serotype specific antibody levels. We recorded only one event each of biopsy proven rejection and de novo development of DSA in our cohort during a follow-up of 18 months after the first vaccination (with PCV13). Given the general risk of alloimmune processes in solid organ transplant recipients, we do not ascribe these events to the administered vaccines [20]. Therefore, we evaluate the sequential administration of both vaccines as immunologically safe in our cohort.

The age of patients in this study ranged from 22 to 76 years, with a median age of 57 years. For patients under and over 65 years of age, the Centers for Disease Control and Prevention (CDC) recommends different vaccination intervals. For younger patients, vaccination intervals vary depending on their specific risk factor, but should not be less than eight weeks between PCV13 and PPSV23. For healthy 65-year-old or older adults, the CDC recommends a minimum interval of one year. However, this can be shortened to a minimum interval of 8 weeks, as is the case in transplant patients with immunodeficiency presented here [21]. In our study, we have chosen a vaccination interval of 6 months, as indicated by the current vaccination recommendation against pneumococci for risk groups of the Robert Koch-Institute in Germany [22].

A common problem, that arises in studies with pneumococcal vaccines, is the lack of a robust threshold to define effectiveness in adults, especially in the case of immunocompromised individuals. The WHO ELISA has been used intensely in evaluation and licensure of pneumococcal vaccines, especially pneumococcal conjugate vaccines in

children. Based on ELISA results from three clinical studies on the seven-valent pneumococcal conjugate vaccine (Prevenar), the WHO defined a correlate of protection of 0.35 µg/mL for serotype-specific antibody concentrations for the license of vaccines against invasive pneumococcal disease in children [23]. But this threshold does not aim to imply protective status in an individual, nor could it be used to assume protection against other pneumococcal infections like pneumonia or otitis media, which may require higher antibody levels [24]. Other authors suggested the aforementioned cut-off should be higher [8, 25, 26]. In our study, we observed that for some serotypes, e.g. serotype 14, the baseline antibody concentrations were already much higher than 0.35 µg/mL.

Therefore, we tend to focus more on the relative increase of antibody levels after vaccination in comparison to baseline values as it was also done in a phase III clinical trial to evaluate the safety and immunogenicity of PPSV23 [15] and in a previous study in KTR investigating the immunogenicity of a sequential vaccination with PCV7 and PPSV23 [26]. The vaccination regiment in the current study comprises two different vaccine types, one polysaccharide vaccine (PPSV23) and one conjugate vaccine (PCV13). Polysaccharide antigens are large molecules consisting of repetitive epitopes. These molecules are not processed by antigen-presenting cells and interact directly with B cells, inducing antibody responses in the absence of T cells. However, T cell-independent responses have several limitations, including poor induction of immunological memory. In contrast, antibody responses against protein antigens are T cell-dependent and result in long-lived immunity due to the generation of immunological memory. Pneumococcal polysaccharide vaccines elicit responses that mainly induce IgG2 in adults, whereas both IgG1 and IgG2 responses are induced by pneumococcal conjugate vaccines [27]. In our study, we could demonstrate that all measured serotype-specific geometric mean antibody concentrations showed more than a twofold increase at month 12 compared to baseline, indicating a sufficient immunogenicity. This includes serotypes, that are an ingredient of only PPSV13 (6A), only PPSV23 (2, 9N, 11A) or both (3, 14). The time courses of serotype 3 and serotype 14 exemplify that the vaccination with PPSV23 hardly boosts the antibody concentrations. Whereas PCV13 doubles them, PPSV23 only slightly increases them. We observed the strongest fold-increase for serotype 9N, which is in line with the mentioned phase III study for PPSV23. The highest absolute antibody concentrations were recorded for serotype 14 as also described in previous studies on different vaccination strategies [8, 26].

Our global anti-pneumococcal antibody data support the courses of serotype-specific antibody concentrations. With exception of IgM (1.7-fold increase), all other antibody subclasses showed more than a twofold increase of GMC at month 12 compared to baseline. We compared our data to the results of a previous study of our group, which comprised a comparable cohort of 47 KTR, who received a single dose of PCV13 [11]. The determination of global antibody concentrations was done with same commercial ELISA in the same lab. The median age of the current cohort was slightly higher (57 years vs. 53 years) whereas the median interval between first vaccination and (last) kidney transplantation was a bit lower (38 months vs. 49 months). Immunosuppressive medication did not deviate significantly as the vast majority in both cohorts received triple therapy with tacrolimus, MPA and corticosteroids. The comparison revealed higher relative increases and absolute values for concentrations of all antibody types at month 12 indicating an enhanced and longer lasting serological immune response after sequential vaccination compared to a single vaccination with PCV13. Our previous study showed that serotype specific IgG antibody concentrations as determined by ELISA correlated significantly with their functional activity measured by the opsonophagocytic assay, indicating that serotype specific IgG antibodies are functional in this immunocompromised cohort [11]. However, it should also be noted that functional antibody concentrations may be lower than the concentration of binding antibodies presented in the current study.

There are only limited data on global and serotype-specific anti-pneumococcal antibody concentrations in healthy adults before and after vaccination and validated protective cut-off values are lacking [28]. Therefore, we compared our data with two groups of healthy adults [18, 19, 29]. The first cohort consists of vaccine naïve healthy individuals [17, 30]. The comparison revealed reduced global antibody concentrations (all subclasses) in KTR before vaccination. One month after first vaccination, comparable antibody concentrations to healthy adults were reached except for IgM. This finding was in line with our previous study on a single administration of PCV 13 in KTR [16]. But 12 months after vaccination, the GMC of all immunoglobulin classes matched or even exceeded those of vaccine naïve healthy adults. This did not apply to the same extent to our previous study [11]. To evaluate serotype-specific antibody concentrations, we compared our patient cohort with healthy adults, that were vaccinated with a single dose of PCV13 [19]. With respect to the tested serotypes, we could only compare antibody concentrations of serotypes 3, 6A and 14. Antibody concentrations were higher for all serotypes in healthy individuals at one month after administration of PCV13. However, at 12 months after vaccination with PCV13 antibody concentrations of serotypes that are also part of PPSV23 were comparable (serotype 3) or even higher (serotype 14) in kidney transplant recipients. In contrast, antibody concentrations of serotype 6A (only part of PCV13) were still much higher in healthy individuals.

Correlation analysis for all antibody subclasses revealed that values before vaccination were predictive of antibody concentrations after vaccination with highest correlation coefficients for IgM antibodies. Overall, the strongest correlation was seen between IgG and IgG2 antibody concentrations, reaching highly significant results ($p < 0.001$) at each timepoint. *Robbins* et al. investigated the immunogenicity of PCV13 in an adult cohort with common variable immunodeficiency (CVID) or IgG subclass deficiency [31]. They observed that higher global IgG and IgG2 values at baseline were associated with protection at one year after vaccination. These findings are in line with a study on patients with systemic lupus erythematodes, for whom higher global IgG2 serum concentrations were associated with long-lasting protection three years after sequential PCV13/PPSV23 vaccination [32]. In general, IgG2 is known to play a key role in the defense against pneumococcal infections [33].

Decreased anti-pneumococcal IgA and IgM levels have been observed in healthy adult blood donors [18], but have also been associated with a pronounced rate of respiratory infections in patients with CVID [34] and primary antibody deficiency (PAD) [35]. It is also known that individuals characterized as having an intact humoral response based on measurement of serotype-specific IgG concentration can still display impaired anti-pneumococcal IgM and IgA levels [36]. Thus, an additional determination of anti-pneumococcal IgM and IgA concentrations could yield more precise information on the humoral response to pneumococcal vaccines in individuals, but the clinical benefit is questionable [36].

The global anti-pneumococcal IgG ELISA shows a good correlation to the GMC of serotype-specific antibodies, with exception of serotype 2, for most time-points in our study and was also reported previously [11]. This commercial assay therefore serves as a cost-effective and easy tool to monitor the humoral immune response to pneumococcal vaccination in clinical routine [28]. But to get more insight into serotype-specific serological responses, especially in the case of low-level global anti-pneumococcal IgG concentrations, serotype-specific WHO ELISA is required [28]. In particular, serotype 3 remains a dominant cause of invasive pneumococcal disease (IPD) in Europe [37]. In our study, only 56.5% of recipients showed a twofold increase of antibodies against serotype 3 after 12 months, compared to baseline. This is approximately 10% less than the mean value for the other serotypes. Moreover, concentrations were by far the lowest of the tested serotypes. Since serotype 3 is a main driver of IPD in Germany, these results corroborate the well-known lack of vaccine-protection [37–39]. However, for a more accurate assessment of this problem, a longer follow-up of recipients and larger group of participants are necessary.

We compared the vaccine-induced immune responses with clinical patient data. We observed that patients with MPA treatment had significantly lower global IgG and IgG2 antibody concentrations compared to patients without MPA intake. We also tested, if we could confirm this finding for serotype-specific antibodies, but although we did see the same trend, it did not reach significance (data not shown). This may be explained by the small size of both groups ($n = 33$ vs. $n = 13$) in the sense that statistical significance was only reached by a summed effect of global antibody concentrations. Interval between (last) kidney transplantation and first vaccination were the only other variable with a significant association with global antibody GMC. In that case we recorded a positive correlation meaning that a longer interval, which normally implicates reduced immunosuppressive treatment, was associated with higher IgG and IgG2 GMCs. However, regression analysis revealed that only MPA treatment proved to be significant for IgG and IgG2 GMC at month 7 and month 12 after first vaccination, highlighting its relevance in case of long-term vaccine immunogenicity. This is in line with previous studies, which generally describe the dominant effect of MPA treatment on humoral immune responses [40] or specify its impact on serological responses after pneumococcal vaccination [11]. Mycophenolic acid inhibits the generation of guanine nucleotides. Unlike different other cell types (e.g., neurons, hepatocytes and renal cells), lymphocytes can only generate guanine nucleotides de novo. Therefore, they are a rather specific target of MPA, which leads to reversible inhibition of B and T cell proliferation. This explains the negative effect on humoral immune responses as described previously and in the current study. The calcineurin inhibitor tacrolimus primarily affects T cell functionality but can indirectly inhibit B cell functions that depend on $CD4^+$ T cell interaction. Accordingly, tacrolimus is more likely to impair the efficacy of T cell-dependent conjugate vaccines like PCV13 than T cell independent polysaccharide vaccines. Glucocorticoids, which are also part of the conventional immunosuppressive triple therapy in kidney transplantation, have pro-apoptotic effects on B and plasma cells. The combination of MPA, tacrolimus and glucocorticoids therefore leads to a substantial suppression of humoral immunity [40, 41]. However, our data shows that MPA has the strongest influence on the production of anti-pneumococcal antibodies.

In this subgroup of organ transplant recipients, a third vaccination may be required to reach equivalent antibody concentrations. Pneumococcal booster vaccination has already been shown to improve overall immune protection against pneumococcal disease in immunocompromised patients such as HIV-positive adults and is highly recommended for adults with chronic obstructive pulmonary disease [42, 43]. Further studies are necessary to determine the most suitable vaccine for booster vaccination in

kidney transplant recipients and to compare its impact on the incidence of pneumococcal disease.

There are several limitations to the study that should be acknowledged. First, the study lacked a control group that received the same vaccine sequence, which would have allowed a more robust comparison of the effectiveness and safety of the PCV13 and PPSV23 sequential vaccination. Direct comparison of PPSV23 vaccine efficacy between KTR and the healthy blood donor population is not ideal. Second, the follow-up period of 18 months may not be sufficient to assess the long-term effectiveness. Vaccine efficacy may decrease after 5 years, so CDC recommends booster vaccination after 5 years for adults 65 years and older, who received PCV13 at any age and PPSV23 before age 65 [21]. Third, the study was conducted in a single center in Germany, which may limit the generalizability of the findings to other regions or populations with different characteristics, prevalent serotypes or risk factors. However, we found that all measured serotype-specific geometric mean antibody concentrations showed more than a twofold increase at month 12 compared to baseline, indicating sufficient immunogenicity of sequential vaccination in KTR. In addition, diminished antibody rise was narrowed down to therapy with MPA.

To conclude, sequential vaccination with PCV13 and PPSV23 in kidney transplant recipients results in a superior antibody response compared with single vaccination. MPA treatment significantly reduced the antibody response in contrast to any other immunosuppressive therapy.

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Data availability The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any financial or non-financial relationships that could be construed as a potential conflict of interest.

Institutional review board statement The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the University Hospital Essen, Germany (14–5858-BO).

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Article

Correlation of Fc Receptor Polymorphisms with Pneumococcal Antibodies in Vaccinated Kidney Transplant Recipients

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Abstract: Several polymorphisms within Fc receptors (FCR) have been described, some of which correlate with allograft function. In the current study, we determined three Fcγ receptor and five Fcα receptor dimorphisms in 47 kidney transplant recipients who had been vaccinated against *Streptococcus pneumoniae*. We analyzed if FCR genotypes correlated with pneumococcal antibodies and their serotype-specific opsonophagocytic function, tested prior to and at months 1 and 12 post-vaccination. In parallel, we assessed antibodies against HLA and MICA and determined kidney function. We observed that IgG2 antibodies against pneumococci at months 1 and 12 after vaccination and IgA antibodies at month 1 differed significantly between the carriers of the three genotypes of FCGR3A rs396991 (V158F, $p = 0.02$; 0.04 and 0.009, respectively). Moreover, the genotype of FCGR3A correlated with serotype-specific opsonophagocytic function, reaching statistical significance ($p < 0.05$) at month 1 for 9/13 serotypes and at month 12 for 6/13 serotypes. Heterozygotes for FCGR3A had the lowest antibody response after pneumococcal vaccination. On the contrary, heterozygotes tended to have more antibodies against HLA class I and impaired kidney function. Taken together, our current data indicate that heterozygosity for FCGR3A may be unfavorable in kidney transplant recipients.

Keywords: Fcγ receptor polymorphism; Fcα receptor polymorphism; *Streptococcus pneumoniae*; vaccination; kidney transplantation; pneumococcal antibodies; serotype-specific opsonophagocytic function; HLA antibodies

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1. Introduction

Infection and rejection are two major obstacles in transplantation medicine. Both are mediated by humoral and cellular immune responses, which are suppressed after transplantation. Antibodies against microorganisms and allografts bind to Fc receptors and can thereby lead to immune activation. A polymorphism within the Fcγ receptor (FCGR), FCGR2A rs1801274 [1], is correlated with the recurrence of acute otitis media after infection with *Streptococcus pneumoniae* (*S. pneumoniae*) [2] (Table 1). *S. pneumoniae* is a gram positive bacterium that frequently colonizes the human nasopharynx [3]. Outside the nasopharynx, it can cause lobar pneumonia, meningitis, otitis media, or sinusitis, and it is especially

harmful after coinfection with the influenza virus [4]. A severe form of infection is invasive pneumococcal disease (IPD), which has a fatality rate of approximately 10% [3,5,6]. According to data by the Centers for Disease Control and Prevention, the rate of IPD in organ transplant recipients is 25 times greater than in the general population [5,6]. Vaccination against *S. pneumoniae* is recommended in individuals with immunocompromising conditions because it has been shown to reduce the incidence of IPD [7–9].

There is already significant data on the associations between FCGR polymorphisms and transplant outcome [10–14]. Three FCGR polymorphisms may be predictive of graft survival, rejection, and HLA antibodies after solid organ transplantation or of immune recovery after hematopoietic stem cell transplantation (FCGR2A rs1801274 [10,11], FCGR3A rs396991 [15], and FCGR3B rs35139848 [16]) (Table 1).

Table 1. Previous functional data on Fcγ receptor polymorphisms.

Fc Receptor	SNP (Substitution)	Amino Acid	Function	Reference
FcγRIIa	FCGR2A rs1801274 (A vs. G)	H131R	R131 homozygous: • Recurrence of acute otitis media after infection with *Streptococcus pneumoniae* ↑ • Graft survival ↓ • Acute rejections ↑	 [2] [10] [11]
FcγRIIIa	FCGR3A rs396991 (G vs. A)	V158F	Affects the affinity of IgG1 to IgG4 and influences immune cell activation V158 carriers: • Peritubular capillaritis ↑ • IFN-γ ↑ after HLA antibody stimulation	[17–21] [15]
FcγRIIIb	FCGR3B rs35139848 (A vs. G)	(Intron)	Neutrophil antigen resulting in the isoform neutrophil antigen 1 (NA1) vs. NA2, affecting N-linked glycosylation of the FcγR Influences neutrophil recovery, severe infections, and transplant-related mortality after hematopoietic stem cell transplantation	[17,19] [16]

SNP—single nucleotide polymorphism.

In previous studies on kidney transplant recipients, we determined pneumococcal immunity prior to and at months 1 and 12 after vaccination with the conjugate vaccine Prevenar (Prevenar 13®, Pfizer, New York, NY, USA). We measured antibodies of the IgG, IgG2, and IgA isotypes and the serotype-specific antibody function by an opsonophagocytic-killing assay (OPA) [22]. In parallel, we assessed IgG antibodies against human leukocyte antigens (HLA) and major histocompatibility complex class I chain-related antigens A (MICA) [23], as it has been debated that vaccination may induce HLA antibodies and allograft rejection [24–30]. We observed that kidney transplant recipients could mount a significant response of pneumococcal antibodies after vaccination, though at a lower level than healthy controls [22]. We furthermore found that in females, but not in males, non-specific HLA-antibodies (i.e., those without donor specificity) increased after vaccination [23]. Apart from IgG antibodies, IgA antibodies against HLA may impact allograft survival [31]. FcαR (CD89) is also capable of triggering IgA-mediated immune responses to pathogens, and it has been proposed to function in circulating IgA clearance [32]. The FcαR gene (FCAR) also contains polymorphic positions.

In the current study, we tested 47 kidney transplant recipients for three FCGR and five FCAR dimorphisms. We analyzed if the respective genotypes correlated with pneumococcal antibodies, with HLA antibodies, or with allograft function.

2. Materials and Methods

2.1. Patients

In total, 47 previously described [23], clinically stable kidney transplant recipients (18 female, 29 male) with a median age of 53 years (range 21–73 years) were included in this prospective, single center study. In addition to pneumococcal antibodies [22] and HLA/MICA antibodies [23], we tested for three FCGR and five FCAR dimorphisms. All patients received a single dose of Prevenar. Blood was drawn immediately prior to vaccination and at months 1 and 12 after vaccination. The median interval between kidney transplantation and vaccination was 49 months (i.e., 4.1 years; range 4 months to 34 years). The median follow-up after vaccination was 51 months (range 44–57 months). The median serum creatinine concentration (range) was 1.7 (0.9–4.9), 1.7 (1.0–5.1), and 1.6 (0.9–4.4) mg/dL pre-vaccination, at month 1, and month 12, respectively, corresponding to an estimated glomerular filtration rate (eGFR) of 42 (13–83), 40 (12–84), and 46 (11–83) mL/min/1.73 m^2, respectively (as determined by the Chronic Kidney Disease Epidemiology Collaboration formula [33]). Ten patients were treated with cyclosporine A, 31 with tacrolimus, 34 with mycophenolate mofetil (MMF), 44 with prednisone, four with everolimus, and two with eculizumab. Thus, the majority was treated with tacrolimus, MMF, and prednisone. This study was approved by the institutional review board of the University Hospital Essen (14-5858-BO) and written informed consent was obtained from all participants. It was carried out in accordance with the Declarations of Helsinki and Istanbul and their latter amendments.

2.2. Vaccine

The 13-valent pneumococcal vaccine Prevenar contains the polysaccharides of 13 pneumococcal serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F), individually conjugated to a nontoxic mutant form of diphtheria toxin cross-reactive material 197 (CRM197).

2.3. Genotyping of FCAR and FCGR Polymorphisms

The genotyping of three FCGR polymorphisms (FCGRIIA (rs1801274, 519A/G, codon 131 histidin to arginine), FCGRIIIA (rs396991, 559G/A, codon 158 valin to phenylalanin) and FCGRIIIB (rs35139848, neutrophil antigen 1/2 (NA1)/NA2)), four intron polymorphisms (rs10402324, rs11084377, rs1865097, and rs4806608), and one exon 5 polymorphism (rs16986050, 844A/G, which changes codon 248 from AGC (Serin) to GGC (Glycin) in the cytoplasmic domain of the receptor) of the FCAR on chromosome 19 was carried out in a StepOnePlus real-time PCR detection system (Applied Biosystems, Darmstadt, Germany) using TaqMan SNP Genotyping Assay and TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Darmstadt, Germany). A TaqMan MGB probe labelled with VICTM dye detected allele 1 and a probe labelled with FAMTM dye detected allele 2.

2.4. Determination of Antibodies against Pneumococci

Antibodies against *S. pneumoniae* were determined by three ELISA formats which detect IgG, IgG2, or IgA antibodies against 23 pneumococcal serotypes (VaccZyme™, The Binding Site, Schwetzingen, Germany) [22]. The assay was performed according to the manufacturer's instructions.

Moreover, the serotype-specific functional response was measured by a validated killing assay (OPA), as published previously [22,34]. Briefly, heat-inactivated sera were serially diluted. Target bacteria were added to assay plates and incubated for 30 min at 25 °C. Baby rabbit complement (3–4 weeks old, Pel-Freez Biologicals, Rogers, AR, USA, 12.5% final concentration) and differentiated HL-60 cells (ATCC CCL-240) [34,35] were then added to each well at an approximate effector-to-target ratio of 200:1 or 400:1, depending on serotypes. These HL-60 cells were differentiated into granulocytes using 100 mM dimethylformamide (DMF). The viability of the differentiated HL-60 cells was assessed by trypan blue exclusion and annexin V/propidium iodide staining. The HL-60 cells were incubated with DMF

for at least 3–4 days and used only if CD35 (complement receptor 1) expression was up-regulated by ≥ 55% of the cell population and CD71 (transferrin receptor) expression was down-regulated by ≤ 15% of the cell population, as assessed by flow cytometry. Moreover, HL-60 cells express low levels of FcγRIII (CD16). Unfortunately, we have no information on the FCGRIIIA V158F polymorphism; however, it is known that HL-60 cells are homozygous for the arginine R131 allele of FCGRIIA [36]. The assay plates with effector and target cells were incubated for 45 min at 37 °C on a shaker. Thereafter, the reaction was stopped and a 10 µL aliquot was transferred to each well of a MultiScreen HTS HV filter plate (Millipore, Livingston, UK), applying vacuum. Then 150 µL of HySoy medium was added to each well and filtered through. The filter plates were incubated at 37 °C with 5% CO2 overnight, then fixed with Destain Solution (Bio-Rad, Watford, UK). Thereafter, the plates were stained with Coomassie Blue and destained once. Colonies were enumerated on a Cellular Technology Limited (CTL) ImmunoSpot Analyzer® (Cleveland, OH, USA). The OPA is generally accepted as the best functional correlate of pneumococcal immunity [34,37]. The interpolated reciprocal serum dilution that resulted in complement-mediated killing of 50% of the bacteria was defined as the OPA titer. The lower limit of quantitation (LLOQ) was 1:8 and was determined during assay validation for each serotype. Values below the LLOQ were defined as half of the LLOQ (1:4). Serum samples were tested by OPA at the Pfizer Vaccines Research Laboratory (New York, NY, USA) for all 13 serotypes included in the Prevenar vaccine.

2.5. Determination of HLA and MICA Antibodies

All samples were tested for IgG antibodies against HLA class I and class II and MICA using Luminex™ technology-based assays (LABScreen™ Mixed Beads, One Lambda/Thermo Fisher, Canoga Park, CA, USA) according to the manufacturer's instructions and as described in detail previously [23]. In brief, the responses towards each bead were scored as 8 (positive), 4 (undetermined), or 1 (negative), and the respective responses were summed up. Thereby, we obtained antibody score values for HLA class I and class II and MICA of 12–96, 5–40, and 2–16, respectively.

2.6. Statistical Analysis

Data were analyzed using GraphPad Prism version 8.4.2 for Windows (GraphPad Prism Software, La Jolla, CA, USA) or IBM SPSS Statistics version 23 (Armonk, NY, USA). The correlation of FCR genotypes with pneumococcal or HLA and MICA antibodies was analyzed by the Kruskal–Wallis test with Dunn's multiple comparisons test. The correlation of FCR genotypes with allograft function or with immunosuppressive treatment was analyzed by contingency tables (chi-square test or Fisher's exact test, as appropriate). Moreover, a Bonferroni correction for multiple testing was applied. As we tested for eight independent FcR polymorphisms, we multiplied the p values by eight. The results were considered significant at $p < 0.05$.

3. Results

3.1. Correlation between Fc Receptor Polymorphisms and Pneumococcal Antibodies

We determined the concentration of IgG, IgG2, and IgA antibodies against *S. pneumoniae* pre- and post-vaccination. Based on the observation that especially soluble FcRs present immunomodulatory properties, for example, the inhibition of B cell proliferation and immunoglobulin production [21,38], we grouped the pneumococcal antibodies by eight FCR genotypes and compared the respective groups of clinically stable kidney transplant recipients by Kruskal–Wallis test. We assessed three time points: prior to vaccination, at month 1, and at month 12. Whereas findings on the FCGR2A polymorphism (FcγRIIa H131R) showed no significant result (Figure 1A), the findings on the FCGR3A polymorphism (FcγRIIIa V158F) reached statistical significance (Figure 1B). IgG2 antibodies against pneumococci at months 1 and 12 after vaccination differed significantly between the groups ($p = 0.02$ and $p = 0.04$), and heterozygous carriers of FCGR3A (genotype AG) had

the lowest antibody concentration. Similarly, the IgA antibodies against pneumococci at month 1 differed significantly ($p = 0.009$). However, after Bonferroni correction for multiple testing, none of the results remained significant. Of note, pneumococcal antibodies prior to vaccination did not differ significantly between the three genotype groups.

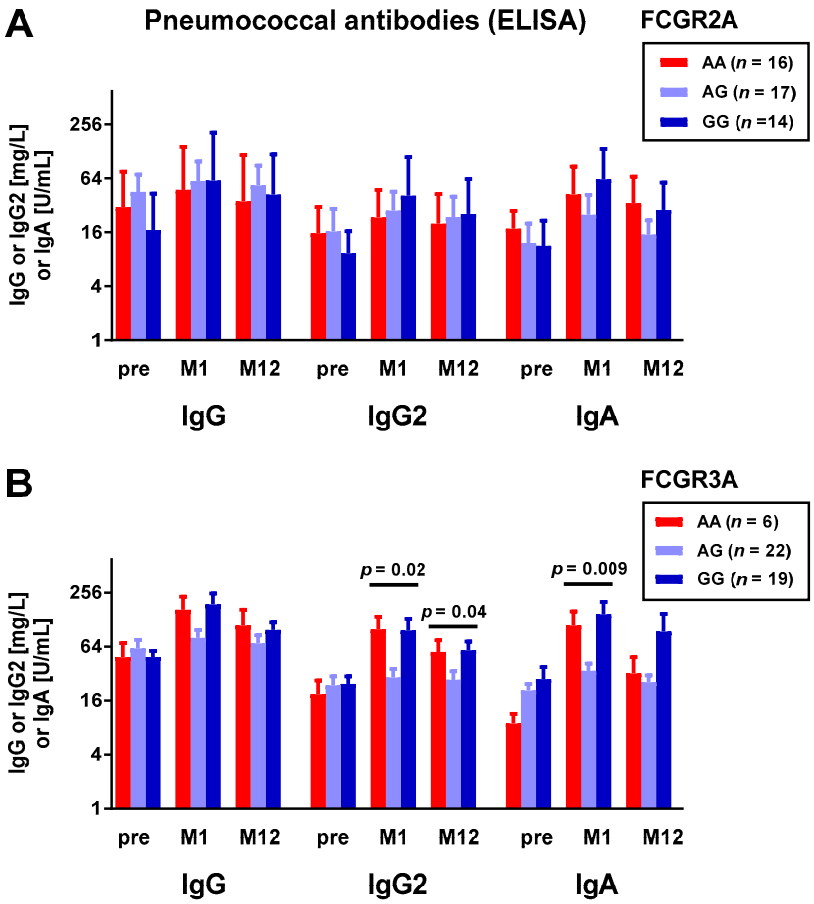

Figure 1. Kidney transplant recipients were genotyped for the polymorphisms FCGR2A rs1801274 (**A**) and FCGR3A rs396991 (**B**). IgG, IgG2, and IgA antibodies determined by commercial ELISA (VaccZyme™) prior to pneumococcal vaccination (pre) and at month 1 (M1) and month 12 (M12) thereafter were grouped by genotype. Antibodies are shown on a logarithmic scale (log2) as geometric means and geometric standard deviation factors [39]. The three groups were compared by Kruskal–Wallis test with Dunn's multiple comparisons test. Of note, none of the results remained significant after Bonferroni correction.

We furthermore considered antibody function as determined by serotype-specific OPA. This assay is a measure not only of the antibody concentrations, but also of their binding to Fc receptors. Similar to the ELISA data, serotype-specific OPA results were significantly dependent on the FCGR3A genotype (Figure 2). In detail, differences between genotypes at month 1 after vaccination reached statistical significance ($p < 0.05$) in 9 out of 13 serotypes tested (1, 3, 4, 6A, 6B, 7F, 9V, 18C, and 23F). At month 12, differences between genotypes were still significant for 6 out of 13 serotypes (1, 3, 6A, 6B, 7F, and 18C). After Bonferroni correction, at month 1, three results remained significant (for serotype 1, 6A, and 6B), and at month 12, four remained significant (for serotype 1, 3 6A, and 7F). The remaining FCAR and FCGR polymorphisms did not correlate significantly with the pneumococcal antibodies. Moreover, after vaccination, the heterozygous carriers of FCGR3A (genotype AG) showed only a minor increase in pneumococcal antibody concentration and function, in contrast to the homozygous carriers (AA or GG genotypes).

As there is a known dysregulation of the immune system and functional decline in antibody function with aging, we analyzed our patient cohort separately by age (median 53 years). Overall, data on FCGR3A were similar in younger and older patients, as depicted in Figure 3. In detail, pneumococcal antibodies as determined by ELISA were lower in carriers of the AG vs. GG genotype of FCGR3A, independent of age (Figure 3A,B). As the older cohort comprised only one patient with the genotype AA, a further comparison was not possible. Moreover, the OPA data indicated that carriers of the AG vs. GG genotype had weaker responses after vaccination in both age groups (Figure 3C,D).

3.2. Correlation between Fc Receptor Polymorphisms and HLA and MICA Antibodies

We grouped HLA and MICA antibodies by FCR genotype and compared the respective groups of renal transplant recipients at three time points. The antibody score was used as a measure of the strength of the HLA and MICA antibodies [23], and eight FCR polymorphisms were considered. Using the Kruskal–Wallis test, we observed that HLA and MICA antibody responses did not differ significantly between the carriers of the respective FCR genotypes. However, we observed that the carriers of the AG genotype of FCGR3A tended to show the highest antibody scores for HLA class I (Figure 4A), which fits with impaired allograft function (Figure 4B). This phenomenon occurred irrespective of the time point (both pre- and post-pneumococcal-vaccination).

Furthermore, we analyzed the data set by Fisher's exact test, applying a dominant and recessive model. We observed that in carriers of the (dominant) A allele of FCGR2A, allograft rejection and loss occurred less frequently. When using a dominant model (AA vs. AG plus GG) we found an odds ratio (OR) of 0.6 and 0.0, respectively (Table 2). Moreover, carriers of the GG genotype of FCGR3A suffered less frequently from rejection, graft failure, and loss. Using a dominant model, differences in the frequency of rejection and graft loss just escaped statistical significance ($p = 0.07$ for both parameters). Of note, both values for the OR were 0.0, pointing to a protective effect of the GG genotype. The frequency of the AG genotype of FCGR3A was doubled in patients with vs. without rejection, graft failure, and loss. Taken together, data on FCGR3A were most consistent and showed by trend that the genotype AG may correlate with impaired kidney function.

Table 2. Kidney allograft function, stratified by the FCGR2A and FCGR3A genotypes.

SNP	Genotype			Model [1]	OR	*p*
FCGR2A rs1801274		**Rejection (*n* = 6)**	**No Rejection (*n* = 41)**			
	AA	1 (17%)	15 (37%)	Dominant	0.6	0.65
	AG	2 (33%)	15 (37%)	Recessive	0.4	0.34
	GG	3 (50%)	11 (27%)			
		Graft Failure (*n* = 6)	**No Graft Failure (*n* = 41)**			
	AA	2 (33%)	14 (34%)	Dominant	1.0	1.00
	AG	2 (33%)	15 (37%)	Recessive	0.8	1.00
	GG	2 (33%)	12 (29%)			
		Graft Loss (*n* = 5)	**No Graft Loss (*n* = 42)**			
	AA	0 (0%)	16 (38%)	Dominant	0.0	0.15
	AG	2 (40%)	15 (36%)	Recessive	0.2	0.15
	GG	3 (60%)	11 (26%)			
FCGR3A rs396991		**Rejection (*n* = 6)**	**No Rejection (*n* = 41)**			
	GG	0 (0%)	19 (46%)	Dominant	0.0	0.07
	AG	5 (83%)	17 (41%)	Recessive	0.7	1.00
	AA	1 (17%)	5 (12%)			

Table 2. *Cont.*

SNP	Genotype			Model [1]	OR	*p*
		Graft Failure (*n* = 6)	**No Graft Failure (*n* = 41)**			
	GG	1 (17%)	18 (44%)	Dominant	0.3	0.38
	AG	5 (83%)	17 (41%)	Recessive	Infinite	1.00
	AA	0 (0%)	6 (15%)			
		Graft Loss (*n* = 5)	**No Graft Loss (*n* = 42)**			
	GG	0 (0%)	19 (45%)	Dominant	0.0	0.07
	AG	4 (80%)	18 (43%)	Recessive	0.5	0.51
	AA	1 (20%)	5 (12%)			

Data were analyzed by Fisher's exact test, using either a dominant or recessive model. [1] Test conditions: allele A (major allele) vs. allele B (minor allele); AA vs. AB plus BB (dominant model); AA plus AB vs. BB (recessive model). SNP—single nucleotide polymorphism; OR—odds ratio.

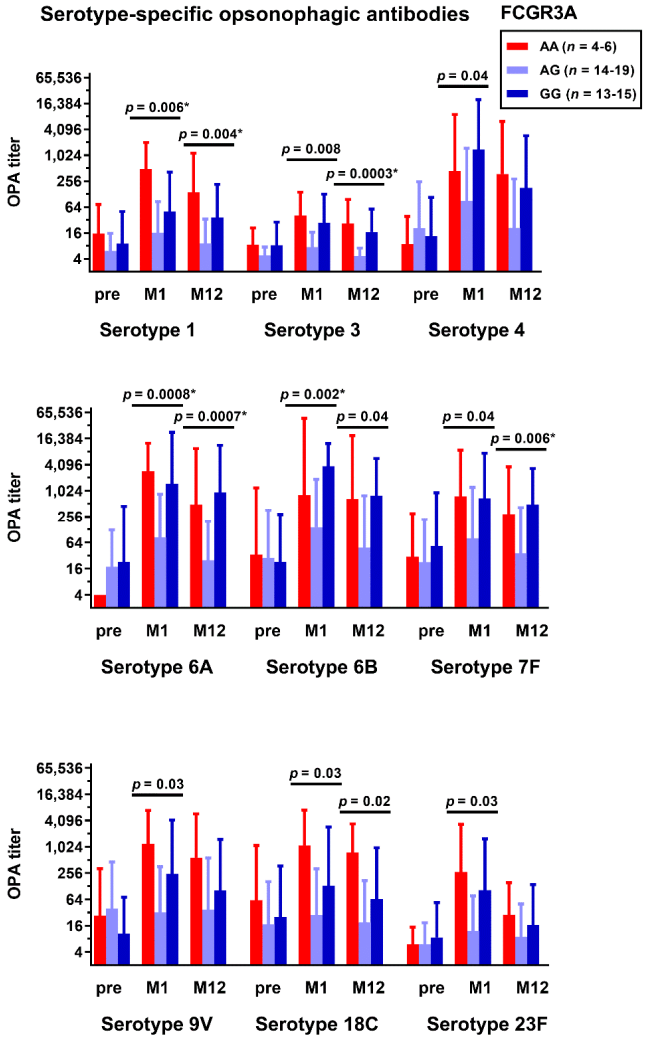

Figure 2. Kidney transplant recipients were genotyped for the FCGR3A rs396991 polymorphism and the serotype-specific opsonophagocytic antibody (OPA) responses, as determined prior to pneumococcal vaccination (pre) and at month 1 (M1) and month 12 (M12) thereafter. The recipients were grouped by genotype. OPA titers are shown on a logarithmic scale (log2) as geometric mean titers and geometric standard deviation factors [39]. The three groups were compared by Kruskal–Wallis test with Dunn's multiple comparisons test. The results that remained significant after Bonferroni correction are marked with an asterisk.

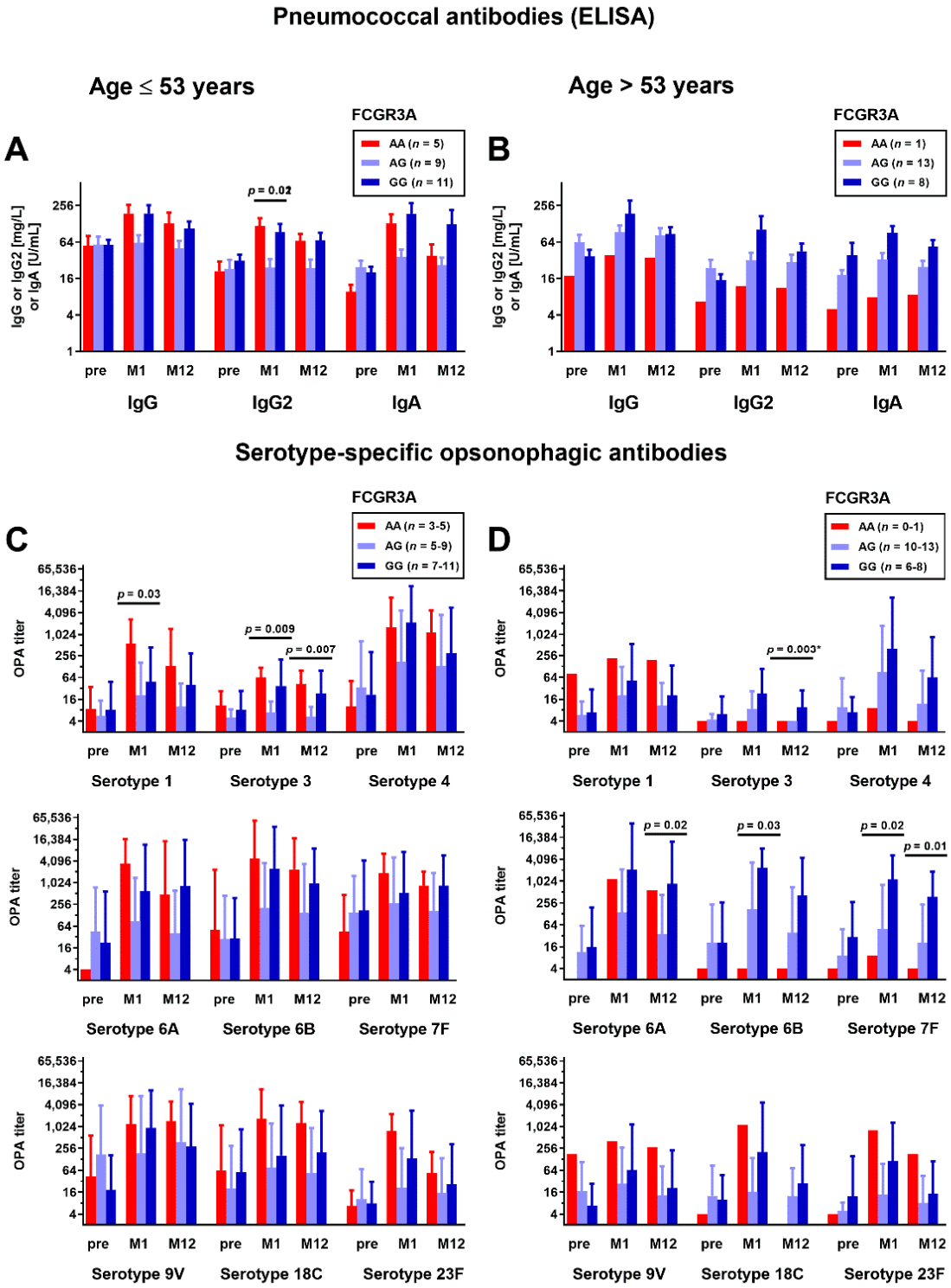

Figure 3. Kidney transplant recipients were genotyped for the FCGR3A rs396991 polymorphism and antibodies determined by commercial ELISA (**A**,**B**), and serotype-specific opsonophagocytic antibody (OPA) responses (**C**,**D**) were divided by genotype and age group. The left panels show data in younger patients (≤ median age of 53 years), while the right panels show data in older patients (>53 years). Antibodies were determined prior to pneumococcal vaccination (pre) and at month 1 (M1) and month 12 (M12) thereafter. The results are shown on a logarithmic scale (log2) as geometric means and geometric standard deviation factors [39]. The three genotype groups were compared by Kruskal–Wallis test with Dunn's multiple comparisons test. The results that remained significant after Bonferroni correction are marked with an asterisk.

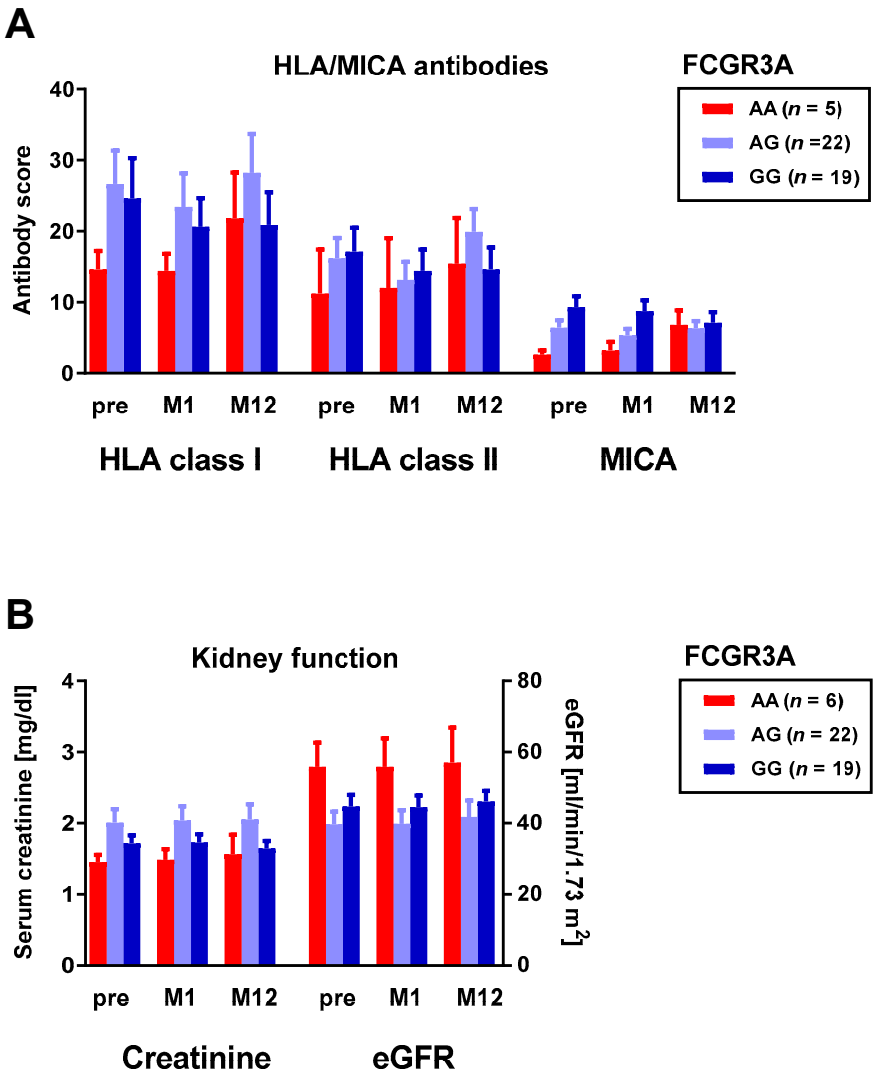

Figure 4. Kidney transplant recipients were genotyped for the FCGR3A rs396991 polymorphism and antibodies and kidney function were determined prior to pneumococcal vaccination (pre) and at month 1 (M1) and month 12 (M12) thereafter. Panel (**A**) shows antibodies against human leukocyte antigens (HLA) and major histocompatibility class I-related chain A (MICA). HLA/MICA antibodies were determined by Luminex™ technology. Responses to individual Luminex™ beads were summed up and an antibody score was generated, as detailed in Section 2. Panel (**B**) indicates serum creatinine concentration and estimated glomerular filtration rate (eGFR). The data represent means and standard errors of the mean (SEM).

3.3. Correlation between Fc Receptor Polymorphisms and Kidney Fucntion

We also analyzed if eight FCR polymorphisms correlated with allograft rejection, failure, and loss. Using a chi-square test, we compared genotype frequencies (AA, AG, and GG) in patients with and without the respective events (Figure 5). We observed that in patients with allograft rejection, the genotype GG of FCGR2A, the genotype AG of FCGR3A, and the genotype AG of FCAR rs10402324 were most abundant (χ^2 = 1.6, 4.8, and 4.2, respectively) (Figure 5A). The frequency of genotype AG of FCGR3A was also enhanced in patients with allograft failure and loss (χ^2 = 3.6 and 3.8, respectively) (Figure 5B,C). Moreover, in patients with allograft loss, the genotype GG of FCGR2A and the genotype GG of FCGRB3B were found more frequently (χ^2 = 3.6 and 2.2, respectively) (Figure 5C). However, most likely because only six patients suffered from rejection and five from graft loss, none of the findings reached statistical significance.

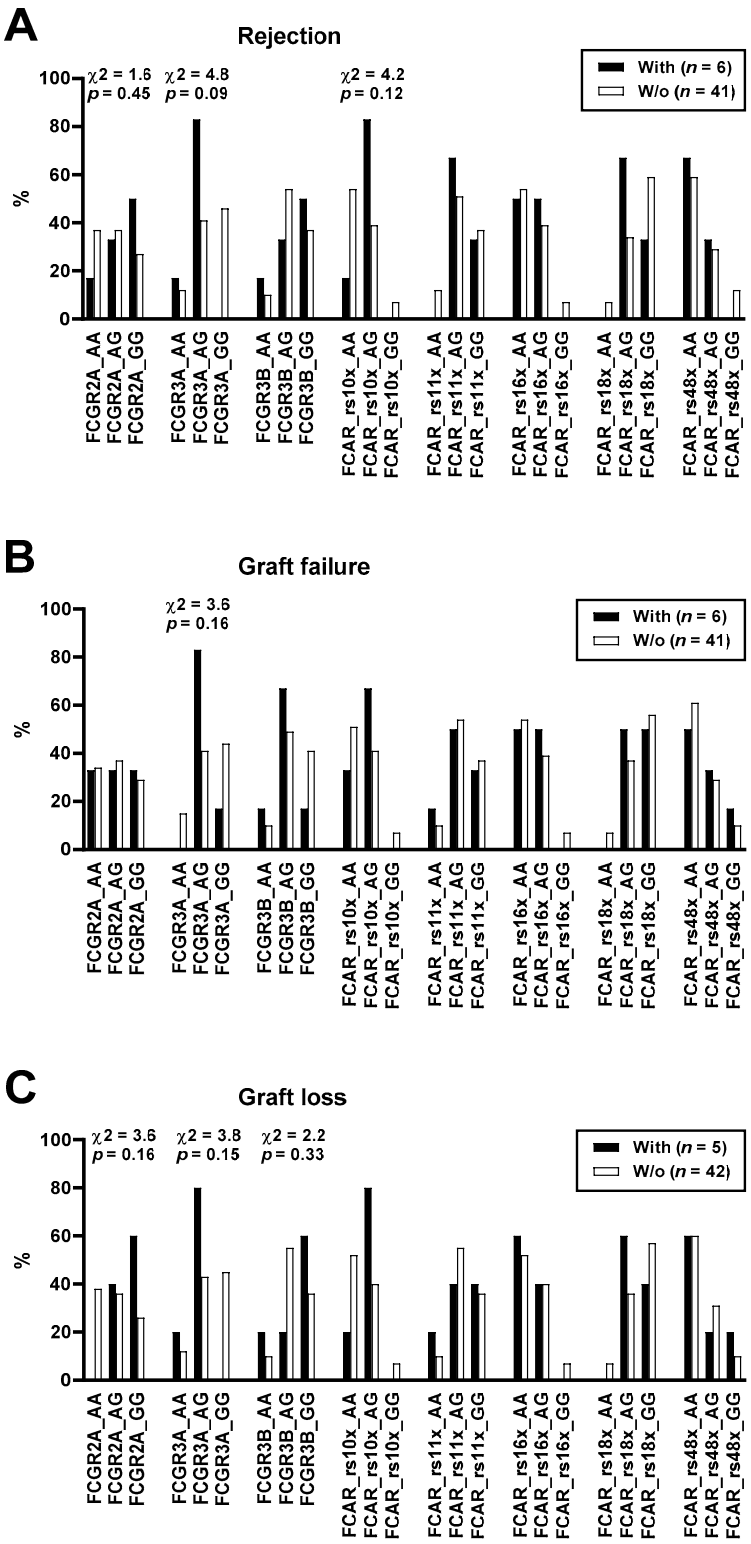

Figure 5. Genotype frequencies of Fcα and Fcγ receptor polymorphisms (FCAR and FCGR, respectively) in 47 kidney transplant recipients with vs. without (w/o) rejection (**A**), graft failure (**B**), and graft loss (**C**). Using a chi-square test, we compared genotype frequencies (AA, AG, and GG). 2A—rs1801274; 3A—rs396991; 3B—rs35139848; rs10x—rs10402324; rs11x—rs11084377; rs16x—rs16986050; rs18x—rs1865097; rs48x—rs4806608.

3.4. Correlation between Fc Receptor Polymorphisms and Immunosuppressive Treatment

After performing Fisher's exact test for the eight FCR polymorphisms and the presence or absence of immunosuppressive drugs as described in the Methods section, only one result reached statistical significance (FCGR2A and cyclosporine A: $p = 0.03$ prior to correction for multiple testing, non-significant after correction). Six out of sixteen patients with the genotype AA of FCGR2A received cyclosporine A, as did four out of seventeen with AG and zero out of fourteen with GG. Nevertheless, carriers of the AA and GG genotypes of FCGR2A showed similar levels of pneumococcal antibodies. However, differences in allograft rejection or graft loss with between the FCGR2A genotype groups could, in principle, be caused by treatment with cyclosporine A. Only six patients suffered from rejection and five from allograft loss, of which three had the genotype GG. Of note, there was no correlation between the FCGR3A polymorphism and immunosuppressive treatment. Due to this small number, the statistical analysis needs to be interpreted with caution.

3.5. Correlation between Kidney Function and Pneumococcal Antibodies

We used the serum creatinine concentration or eGFR as markers of kidney function. Spearman analysis of either serum creatinine or eGFR showed no significant correlation with IgG, IgG2, or IgA antibodies against pneumococci. But–as expected–correlation coefficients with serum creatinine were negative at months 1 and 12 after vaccination (mean values of -0.08 and -0.12, respectively), i.e., lower serum creatinine correlated with higher antibody concentrations. Accordingly, correlation coefficients with eGFR were positive. A similar trend was observed for the OPA results.

4. Discussion

The current study indicates that the genotype AG of FCGR3A rs396991 (FcγRIIIa-V158F) may be unfavorable in kidney transplant recipients. It correlated with significantly lower antibody responses after pneumococcal vaccination and also with an impaired functional capacity of the antibodies, which may cause more infectious complications. Because the HL-60 cell line with its Fc receptors—which remain to be further defined—is the same in all OPA assays in our current study, we hypothesize that the FCGR3A polymorphism correlates with different concentrations of pneumococcal antibodies. The OPA does not include cells with patient-specific FcγR IIIa residues (V158F), and thus we cannot measure directly how this polymorphism affects the binding affinity to the lower hinge region of IgG, directed against pneumococcal antigens. Therefore, because the HL-60 cells are always the same when performing the OPA, the FCGR3A genotype of the HL-60 cells is not expected to have an impact on the OPA results, as it affects the receptor and not the Fc region of IgG, which is patient-specific in the assay. Of note, the HL-60 cells express low levels of FcγRIII, which interact with the Fc region of IgG.

However, FcγR IIIa is also found in a soluble form. It may be assumed that the soluble FcγR IIIa could indirectly impact on antibody production, in line with a recent, comprehensive review [21]. FcγR IIIa was detected in human sera in a soluble form, resulting from proteolytic cleavage by matrix metalloproteinases upon activation of NK cells and neutrophils by protein antigens [21]. Soluble FcγRs presented immunomodulatory properties. For example, they inhibited B cell proliferation and IgM and IgG production [38]. Moreover, crosslinking of activating FcγRs such as FcγR IIIa led to the activation of some nuclear factors such as NFAT, which induces the expression of cytokines important for inflammation and immune regulation, e.g., IL-2, IL-6, IL-8, IL-10, TNF-α, and IFN-γ [40–42]. Finally, the only activating Fcγ receptor on NK cells is FcγRIIIa, which leads after activation to antibody-dependent, cell-mediated cytotoxicity (ADCC). Fcγ receptors expressed on many immune cells are thus capable of inhibiting and activating different cellular responses, which is important not only for controlling microbial infections, but also for regulating immunity [21,43–45]. Previous data show that the dimorphism in the FCGR3A gene affects the affinity to IgG2 and IgG4 [21]. Carriers of the V158 vs. F158 allele of FCGRIII3A showed approximately twofold higher affinity for IgG [17]. FcγR IIIa is expressed on NK cells, T

cells, and subsets of monocytes and macrophages [46]. Because IgG2 and IgA antibody responses after pneumococcal vaccination correlated with FCGR3A genotypes in our study, one may speculate that the effects on antibody concentrations were indirect and most likely by immune regulation. It could be hypothesized that regulatory properties of soluble Fc receptors may be different in heterozygotes for the FCGR3A V158F polymorphism, i.e., they could inhibit the production of immunoglobulins to a higher extent or interact differently with other Fcγ and Fcα receptors, which may then also affect the concentration of IgA.

FcγRs are differentially expressed on immune cells and can be either activating or inhibitory, e.g., with FcγRIIa and FcγRIIIa belonging to the first group [43]. Data on FCGR3A and pneumococcal immunity are not yet published. However, the association of FCGR3A with vaccination-induced antibody responses may be stronger than with FCGR2A [1], as we observed consistent and significant results for FCGR3A. It was previously described that the course of infection with *S. pneumoniae* [1] or with *Plasmodium falciparum* [47] was influenced by the FCGR2A rs1801274 (FcγRIIa-H131R) polymorphism. Of note, IgG2 antibodies exclusively interact with FcγRIIa, and the interaction is mainly restricted to the FcγRIIa-H131 allele [48]. On the contrary, both alleles of FcγRIIa interact with IgG1 and IgG3 [48]. Children homozygous for FcγRIIa-R131 showed a significant increase in recurrence of acute otitis media after pneumococcal vaccination, which consisted of a 7-valent pneumococcal conjugate vaccine (Prevnar 7) and a 23-valent pneumococcal polysaccharide vaccine [1]. Less efficient interaction between pneumococcal antibodies and FcγRIIa was postulated, as the children did not differ in the concentrations of antibodies [1], similar to the current study. Data by the same group indicate that the effective phagocytosis of *S. pneumoniae* depends on FcγRIIa, with significantly higher phagocytosis levels in HH homozygotes compared to RR carriers [49,50]. In the current study, however, we did not observe an influence of FcγRIIa on functional antibody responses, as determined by OPA. The effect may be masked as vaccination led to an increase in (total) IgG, IgG2, and IgA antibodies [22], and the OPA measures all functionally active antibodies and not solely IgG2.

Furthermore, carriers of the AG genotype of FCGR3A showed, by trend, more IgG antibodies against HLA and MICA and inferior graft function. Of note, the presence of donor-specific IgG antibodies against HLA is an established risk factor for allograft survival [51]. Data on an intronic dimorphism in the FCGR3B gene were less clear. This dimorphism (also referred to as neutrophil antigen 1 vs. 2) affects N-linked glycosylation of the FcγR and, thereby, receptor function [17,19]. Whereas a study on 56 pediatric kidney transplant recipients did not observe a significant correlation between the FCGR3A and FCGR3B dimorphisms and allograft rejection [11], a study on 85 adult kidney transplant recipients with donor-specific HLA antibodies—who were recruited upon the antibody screening of 741 prevalent patients—found a higher rate (and extent) of peritubular capillaritis in protocol biopsies in homozygous and heterozygous carriers of FcγRIIIa-V158 (the FCGR3A genotypes AA and AG) [15]. NK92 cells expressing FcγRIIIa-V158 produced significantly more interferon-γ upon incubation with HLA antibody-coated cells than those expressing FcγRIIIa-F158 [15]. Moreover, it was described that the FcγRIIIa-F158V polymorphism influenced the efficacy of depleting antilymphocyte antibodies [52]. Thus, the protective effect of FcγRIIIa-F158 (the FCGR3A genotype GG) against rejection and allograft loss is in line with the previous literature [15]. Extending these previous data, we observed that especially heterozygous carriers of FcγRIIIa-V158 (the FCGR3A genotype AG) may be at risk of allograft rejection and loss. Furthermore, in accordance with that large previous study [15], the genotypes of FCGR2A and FCGR3B did not correlate significantly with allograft function.

Our study has several limitations. As the cohort was rather small, the results should be confirmed by larger independent studies. Specifically, the findings on the pneumococcal antibody ELISA need to be repeated. Moreover, we unfortunately have no information on the FCGR3A genotype of the HL-60 cell line used for the OPA. Genotyping for FCGR polymorphisms is thus one of the next steps. Finally, this study does not include an analysis

of the potential mechanisms of how the polymorphism impacts the antibody concentration or function, which has to be further analyzed.

5. Conclusions

This study is the first showing that FCGR3A rs396991 (FcγRIIIa-V158F) impacts the efficacy of pneumococcal vaccination. A deeper understanding of this effect might help to optimize vaccination programs (e.g., the timing or an increased dose of the vaccine) in order to ensure a sufficient strength of immune responses, especially in sensitive groups such as transplant recipients.

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Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Article

Establishment of an ELISpot Assay to Detect Cellular Immunity against *S. pneumoniae* in Vaccinated Kidney Transplant Recipients

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Abstract: In organ transplant recipients, the rate of invasive pneumococcal diseases is 25 times greater than in the general population. Vaccination against *S. pneumoniae* is recommended in this cohort because it reduces the incidence of this severe form of pneumococcal infection. Previous studies indicate that transplant recipients can produce specific antibodies after pneumococcal vaccination. However, it remains unclear if vaccination also induces specific cellular immunity. In the current study on 38 kidney transplant recipients, we established an interferon-γ ELISpot assay that can detect serotype-specific cellular responses against *S. pneumoniae*. The results indicate that sequential vaccination with the conjugated vaccine Prevenar 13 and the polysaccharide vaccine Pneumovax 23 led to an increase of serotype-specific cellular immunity. We observed the strongest responses against the serotypes 9N and 14, which are both components of Pneumovax 23. Cellular responses against *S. pneumoniae* correlated positively with specific IgG antibodies (r = 0.32, p = 0.12). In conclusion, this is the first report indicating that kidney transplant recipients can mount specific cellular responses after pneumococcal vaccination. The ELISpot we established will allow for further investigations. These could help to define, for example, factors influencing specific cellular immunity in immunocompromised cohorts or the duration of cellular immunity after vaccination.

Keywords: pneumococcal conjugate and polysaccharide vaccines; sequential vaccination; kidney transplant recipients; serotype specific cellular immunity; interferon-γ ELISpot

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1. Introduction

The gram-positive bacterium *Streptococcus pneumoniae* (*S. pneumoniae*) frequently colonizes the human nasopharynx [1]. Outside the nasopharynx, it can lead to lobar pneumonia, meningitis, otitis media, or sinusitis. Apart from local infection, it can cause invasive pneumococcal diseases (IPD), which has a fatality rate of approximately 10% [1–3]. According to data by the Centers for Disease Control and Prevention, the rate of IPD in organ transplant recipients is 25 times greater than in the general population [2,3]. Vaccination against *S. pneumoniae* is recommended in individuals with immunocompromising conditions because it has been shown to reduce the incidence of IPD [4–6].

Apart from polysaccharide vaccines against *S. pneumoniae* (e.g., Pneumovax 23, MSD Sharp and Dohme, Haar, Germany), there are vaccines conjugated to a nontoxic mutant form of diphtheria toxin (e.g., Prevenar 13, PCV13, Pfizer, New York, NY, USA) [7], that act

T-cell-dependently. In Germany, a sequential administration of the 13-valent pneumococcal conjugate vaccine followed by the 23-valent pneumococcal polysaccharide vaccine after 6–12 months is recommended for immunocompromised individuals such as transplant recipients [8]. Vaccinees first receive the glycoconjugate vaccine Prevenar 13. According to previous data in mice, CD4+ T cells could recognize glycan-modified peptides presented by major histocompatibility complex (MHC) class II (carbohydrate-specific helper CD4 T+ cells, Tcarbs) [9,10]. These specific Tcarbs could enhance the production of class-switched (IgG) antibody responses directed against pneumococcal polysaccharides. However, these previous data are not able to determine the source of interferon (IFN)-γ secretion in our ELISpot assays, where cells were stimulated by (non-conjugated) polysaccharide antigens.

Serological control of vaccination responses is recommended in immunocompromised patients, although it remains unclear as to what extent antibody titers reflect protection [11]. The protection achievable by this vaccine regimen remains unclear in transplant cohorts [12–14]. There is currently only data on specific humoral immunity after vaccination against *S. pneumoniae* [12–16]. Specific T-cell data after vaccination against pneumococci are not yet published in a transplant cohort. However, in healthy adults, it could be shown that cellular immunity towards pneumococcal polysaccharides was increased by vaccination [17].

The aim of the current study was to establish an ELISpot that is sensitive enough to detect specific cellular immunity against *S. pneumoniae* in vaccinated kidney transplant recipients.

2. Materials and Methods

2.1. Patients

In total, 38 clinically stable kidney transplant recipients (70 samples) were included in this cross-sectional, single-center study (Table 1). The median age was 53 years (range 23–77 years); 12 patients were female and 26 were male. The patients received two vaccinations against *S. pneumoniae.* They were vaccinated sequentially, with a single dose of Prevenar 13, followed by a single dose of Pneumovax 23 six months later. The median interval between the (last) kidney transplantation and the first vaccination was 38 months (3 months–33 years).

Table 1. Characteristics of 38 kidney transplant recipients vaccinated against *S. pneumoniae*.

Parameter	Median (Range) or Number (No.)
Median age (range), years [1]	53 (23–77)
Patient sex (female/male)	12/26
Median interval TX-vaccination (range), months	38 (3–395)
Median serum creatinine (range), mg/dL	
Pre vaccination	1.6 (0.9–3.6)
Month 6 post vacc.	1.5 (0.6–3.7)
Month 12 post vacc.	1.6 (0.9–3.9)
Immunosuppression, no. [1]	
Cyclosporine A	5
Tacrolimus	28
Mycofenolate mofetil	21
mTOR inhibitors	6
Corticosteroids	36
Belatacept	2
Kidney transplantation, no.	
First	34
Second	4

[1] At the time of the first blood sampling; mTOR—mammalian target of rapamycin.

Stable allograft function (defined as <15% change in serum creatinine concentration within one month prior to vaccination), an interval of ≥3 months to kidney transplantation, and absence of clinical infection, of allograft rejection and of pregnancy were defined as inclusion criteria. Blood samples were drawn immediately prior to vaccination with Pneumovax 23 (month 6), and one month and six months thereafter (months 7 and 12, respectively). This study was approved by the institutional review board of the University Hospital Essen (14-5858-BO), and written informed consent was obtained from all participants. It was carried out in accordance with the Declarations of Helsinki and Istanbul and its subsequent amendments.

2.2. Vaccines

The 13-valent pneumococcal vaccine Prevenar 13 contains polysaccharides of 13 pneumococcal serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19F, 19A, and 23F), individually conjugated to a nontoxic mutant form of diphtheria toxin cross-reactive material 197 (CRM197). The vaccine is formulated in 5 mM succinate buffer containing 0.85% NaCl and 0.02% polysorbate 80, at pH 5.8, and contains aluminum phosphate at 0.125 mg/dose aluminum as an adjuvant. It contains 2.2 μg/dose of each of the serotypes, except for serotype 6B at 4.4 μg/dose (0.5 mL).

The 23-valent vaccine Pneumovax 23 is an unconjugated vaccine that contains 25 μg each of the 23 pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. The vaccine is formulated in phenol and <1 mmol sodium chloride per dose (0.5 mL). Both vaccines were injected into the deltoid muscle.

2.3. Determination of Cellular Immunity against Pneumococci by ELISpot

Nine milliliters of heparinized blood was collected, and peripheral blood mononuclear cells (PBMC) were separated by Ficoll gradient centrifugation. Numbers of PBMC were determined by an automated hematology analyzer (XP-300, Sysmex, Norderstett, Germany). Duplicate or triplicate cultures of 200,000 freshly isolated PBMC were grown without pneumococcal polysaccharides and single cultures with 100, 150, and 200 μg/mL pneumococcal polysaccharides (pneumococcal serotypes (PS) 2, 6A, 9N, 11A, 14, and 25F, all from Pfizer, ATCC, Manassas, VA, USA). The production of IFN-γ was determined using pre-coated ELISpot plates and a standardized detection system (T-Track® ELISpot kit, Mikrogen GmbH, Neuried, Germany; formerly Lophius Biosciences GmbH, Regensburg, Germany). PBMC were incubated without and with pneumococcal polysaccharides in 150 μL AIMV medium (Gibco, Grand Island, NE, USA) at 37 °C. Stimulation with the T-cell mitogen phytohemagglutinin (PHA, 4 μg/mL) served as positive control. Cells were pre-incubated overnight in U bottom plates (BD Falcon, Nijmegen, the Netherlands). Thereafter, they were incubated for further 19 h in the ELISpot plates. These conditions could be defined as optimal. In order to optimize the ELISpot conditions, we titrated the pneumococcal polysaccharides (0.5–800 μg/mL) and performed the cell cultures without and with overnight pre-incubation. Colorimetric detection of cytokine secreting cells was performed according to the manufacturer's instructions. Spot numbers were analyzed by an ELISpot reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). Apart from considering individuals concentrations of the polysaccharides, we determined median values for the optimal concentrations (100, 150, and 200 μg/mL) and subtracted the median of negative controls. Thereby, we generated spots increment, indicating specific spots. Of note, the negative controls reached a mean value of 0.71 spots and a mean standard deviation of 0.47 spots.

2.4. Determination of Cellular Immunity against Pneumococci by Proliferation Assay

In a subset of patients, we also analyzed lymphocyte proliferation, measuring 3H-thymidine uptake. We used 200,000 PBMC per cell culture and stimulated the cells with 50–800 µg/mL of the polysaccharides 2, 6A, 9N, and 14. PBMC were incubated without and with pneumococcal polysaccharides in 150 µL AIMV medium for five days at 37 °C using U bottom plates. Stimulation with PHA was used as positive control. For the last 16 h, the cultures were labeled with 37 kBq 3H thymidine per culture. Cells were then harvested (Harvester 96, Tomtec, Hamden, CT, USA) onto filter pads (Wallac, Turku, Finland), and the incorporated radioactivity was quantified by liquid scintillation counting (1450 Microbeta Trilux, Wallac). Results were expressed as counts per minute. In addition, stimulation indices (SI) were considered (quotient of proliferation with specific stimulation and negative control (proliferation without stimulation)). An SI of at least 3 was defined as positive response.

2.5. Determination of Antibodies against Pneumococci

Antibodies against *S. pneumoniae* were determined by an ELISA that detects IgG antibodies against 23 pneumococcal serotypes (VaccZyme™, The Binding Site, Schwetzingen, Germany). The assay was performed according to the manufacturer's instructions.

2.6. Statistical Analysis

Data were analyzed using GraphPad Prism 8.4.2.679 (San Diego, CA, USA). Data generated without and with pre-incubation and prior to or post vaccination with Pneumovax 23 were compared by the Mann–Whitney *U*-test. Spearman test was used to correlate ELISpot results with numerical variables and Mann–Whitney test to analyze the impact of patient sex on ELISpot results. If not otherwise stated, median values are indicated. Two-sided p values < 0.05 were considered significant.

3. Results

3.1. Optimization of ELISpot Conditions

Initial titration experiments with PBMC from kidney transplant recipients were performed with 0.5–50 µg/mL pneumococcal polysaccharides and showed nearly undetectable cellular responses (Figure 1a), despite vaccination against *S. pneumoniae*. An increase of the polysaccharide concentrations (50–800 µg/mL), combined with an overnight pre-incubation in U bottom plates (Figure 1b,c), led to detectable, dose-dependent cellular responses, reaching a maximum at 100–200 µg/mL.

3.2. Time Course of Pneumococcus-Specific ELISpot Responses

Using the optimized conditions (100, 150, and 200 µg/mL of the polysaccharides and pre-incubation), we tested clinically stable kidney transplant recipients at months 6, 7, and 12 after initiation of vaccination against pneumococci, i.e., we measured the effect of the conjugated pneumococcal vaccine Prevenar 13 at month 6 and the combined effect of both vaccines (Prevenar 13 and Pneumovax 23) at months 7 and 12. Of note, we chose polysaccharide serotypes contained only in the vaccine Prevenar 13 (6A), only in Pneumovax 23 (2, 9N, 11A), in both vaccines (14), or in none of them (25F). The IFN-γ spots detected in our pneumococcus-specific ELISpot assay can be characterized as large and intense (Figure 2).

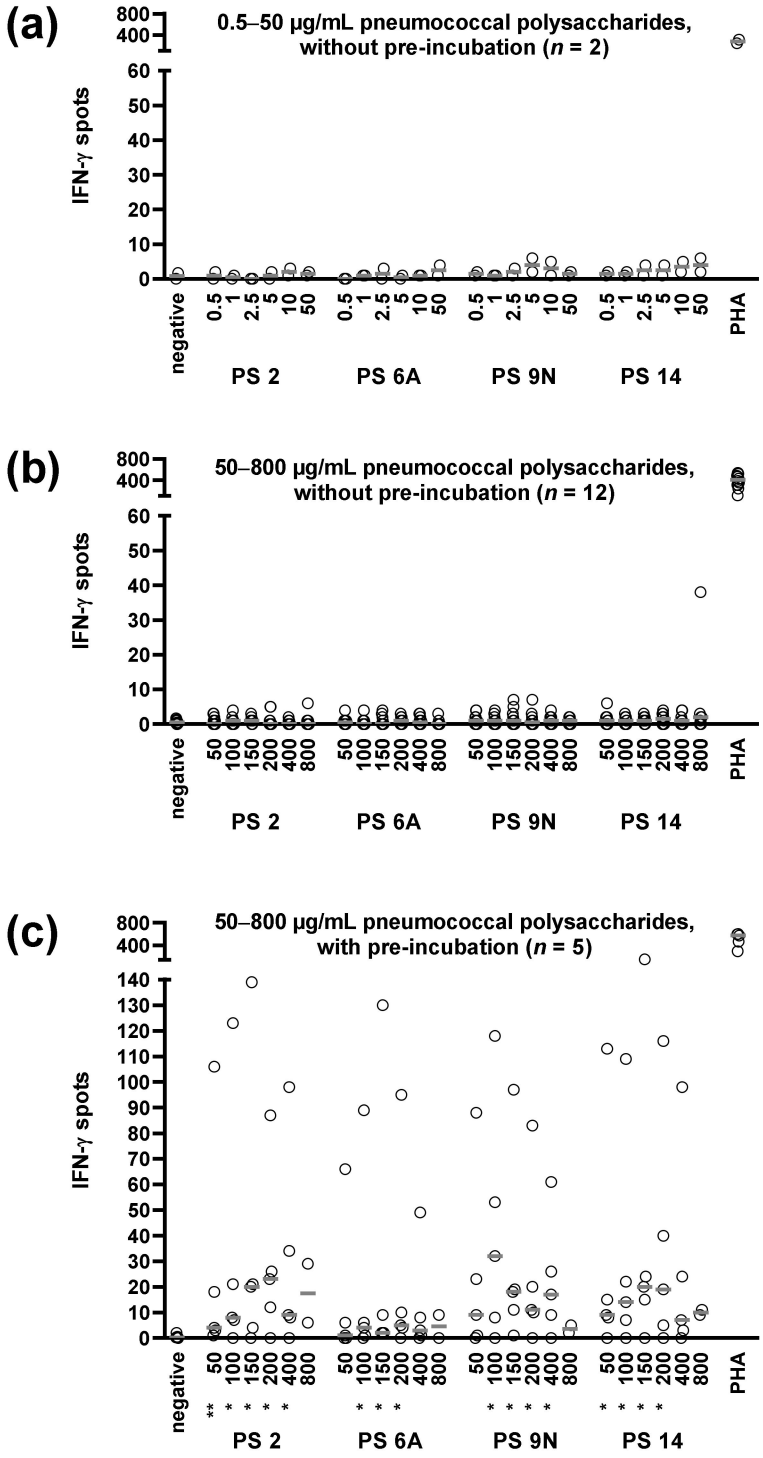
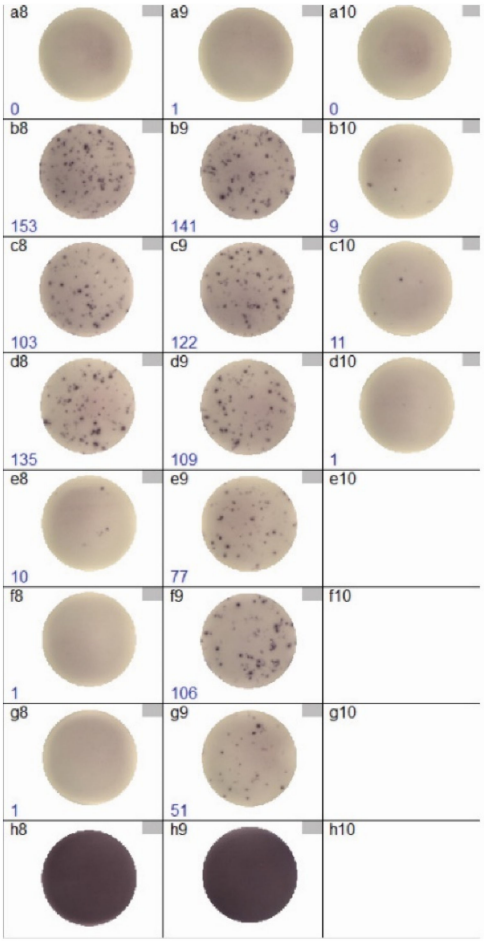


Figure 1. Optimization of ELISpot conditions to determine specific cellular immunity against *S. pneumoniae*. (**a**) The titration of low concentrations of the pneumococcal polysaccharides of the serotypes (PS) 2, 6A, 9N, and 14 (0.5–50 µg/mL), using no pre-incubation step, i.e., the cells were directly incubated in ELISpot plates (without pre-incubation). (**b**,**c**) Results after stimulation with higher concentrations of the pneumococcal polysaccharides (50–800 µg/mL), either without (**b**) or with (**c**) overnight pre-incubation in U bottom plates. In all cases (**a**–**c**), we tested kidney transplant recipients after pneumococcal vaccination. Median values are indicated by grey horizontal lines. Positive control experiments were performed with the T-cell mitogen phytohemagglutinin (PHA). Results as displayed in (**b**,**c**) were compared by Mann–Whitney test. * $p < 0.05$, ** $p < 0.01$.

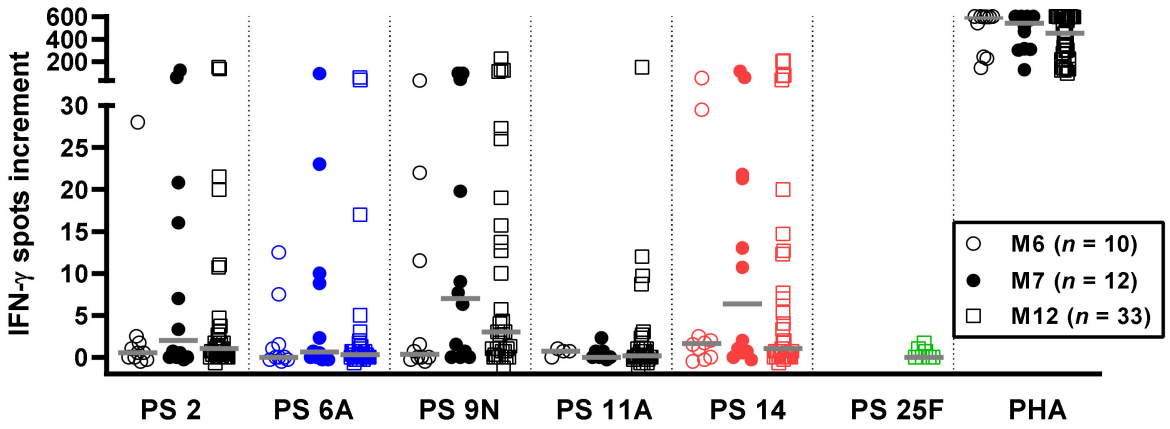


Negative_1	Negative_2	Negative_3
PS 2_200	PS 9N_200	PS 11A_200
PS 2_150	PS 9N_150	PS 11A_150
PS 2_100	PS 9N_100	PS 11A_100
PS 6A_200	PS 14_200	
PS 6A_150	PS 14_150	
PS 6A_100	PS 14_100	
PHA_1	PHA_2	

Figure 2. Interferon-γ ELISpot results (200,000 PBMC/well) in one female, 49-year-old kidney transplant recipient at month 12. The patient had received vaccination with Prevenar 13 and Pneumovax 23 12 months and 6 months prior to bleeding, respectively. Each serotype of the pneumococcal polysaccharides (PS) was used at three concentrations (100, 150, and 200 μg/mL). The left panel shows the ELISpot results, the right panel the plate layout. Confluent spots filling the entire well, as shown in the positive control with phytohemagglutinin (PHA), were set as 600.

For calculation, we used median values for the three concentrations, yielding a single result per sample and time point. For the polysaccharide serotypes 2, 6A, 9N, and 14, we observed an increase of responses at month 7 vs. 6 (Figure 3). This increase appeared to be slightly stronger for three out of four serotypes contained in the vaccine Pneumovax 23 (2, 9N, and 14) than for the serotype 6A, which is contained in Prevenar 13 only. Responses to PS 25F, the serotype that is contained in none of the vaccines, were undetectable at month 12.

Taken together, the data indicate that vaccination with Pneumovax 23 led to an increase of cellular responses to the majority of the serotypes contained in that vaccine. The highest number of specific cells could be detected one month after this vaccination. However, the serotype 11A also contained in Pneumovax 23 did not induce detectable cellular immunity. At month 12 immunity decreased, which is to be expected in the course after vaccination. Unexpectedly, there may also have been a slight increase of responses towards serotype 6A at month 7, which could be explained by cross-reactivity. An absence of cellular responses towards serotype 25F at month 12 met our expectation because it was contained in none of the vaccines.



Prevenar 13	-	+	-	-	+	-
Pneumovax 23	+	-	+	+	+	-

Spots increment						
Median						
M6	0.5	0.0	0.4	0.7	1.6	
M7	2.0	0.6	7.0	0.0	6.4	
M12	1.0	0.3	3.0	0.2	1.0	0.0
Mean						
M6	3.3	2.1	6.6	0.6	9.0	
M7	19.1	11.5	23.2	0.5	20.1	
M12	11.3	3.9	22.5	6.4	20.8	0.5

Figure 3. Specific cellular immunity against *S. pneumoniae* in kidney transplant recipients after having received vaccination with Prevenar 13 (month (M) 6) or Prevenar 13 and Pneumovax 23 (M7 and M12). Of note, we chose pneumococcal serotypes (PS) contained only in the vaccine Prevenar 13 (6A, blue), only in Pneumovax 23 (2, 9N, 11A, black), in both vaccines (14, red), or in none of them (25F, green). Responses towards PS 14 were measured only in 41 out of 55 samples, and responses towards PS 25F were measured only in seven (at M12). We used median values for the three concentrations of each polysaccharide (100, 150, and 200 µg/mL), yielding a single result per sample and time point. Median values are indicated by grey horizontal lines. Positive control experiments were performed with the T-cell mitogen phytohemagglutinin (PHA). Increment means that negative controls were subtracted from results after stimulation with *S. pneumoniae* polysaccharides.

3.3. Concentration Dependency of Pneumococcus-Specific Proliferative Responses

Using 50–800 µg/mL of the pneumococcal polysaccharides, we also performed proliferation assays. Specific responses were weak. In all but one case, counts per minute after stimulation with pneumococcal polysaccharides were below 2200, which we classify as a borderline response (Figure 4). Three out of five vaccinated kidney transplant recipients showed detectable proliferation, as defined by a maximum stimulation index of at least 3. The first patient responded to the serotypes 2, 9N, and 14 (SI of up to 5.8, 3.6, and 3.8, respectively), the second to the serotypes 6A and 9N (SI of up to 3.1 and 5.1, respectively), and the third to serotype 2 (SI of up to 3.9).

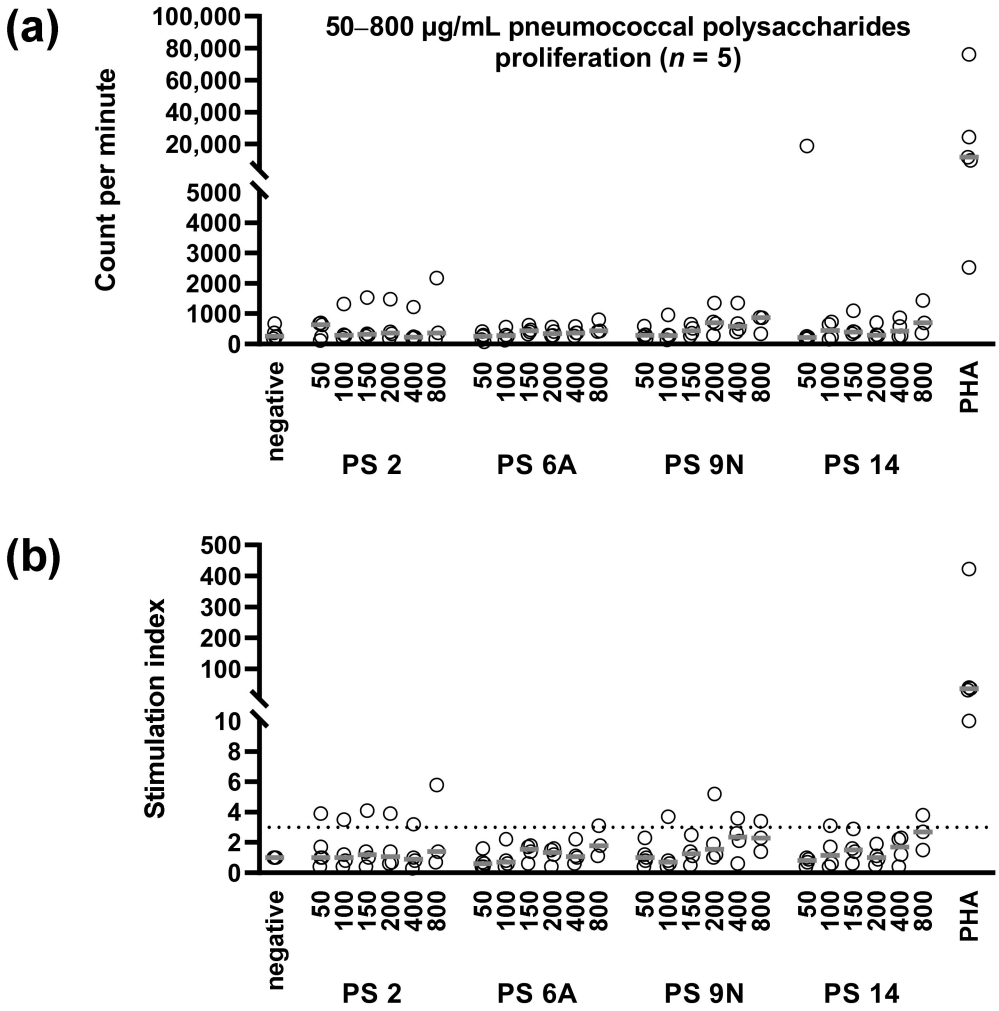


Figure 4. Proliferative responses in vaccinated kidney transplant recipients after stimulation with polysaccharides from *S. pneumoniae*. (**a**) Results given as counts per minute increment; (**b**) stimulation index, i.e., as a quotient of stimulated and unstimulated cultures. We used pneumococcal polysaccharides of serotype (PS) 2, 6A, 9N, and 14 at concentrations of 50–800 µg/mL and cultured cells for six days. Stimulation indices of at least 3 were defined as positive response (dotted line). Median values are indicated by grey horizontal lines. Negative controls were cells cultured without specific stimulation, and positive controls cells were stimulated with the T-cell mitogen phytohemagglutinin (PHA).

3.4. Correlation between Pneumococcus-Specific ELISpot Responses and Specific Antibodies

In parallel to the ELISpot assays, IgG antibodies against 23 pneumococcal serotypes were determined by ELISA. Spearman correlation analysis was performed at month 12 after vaccination and considered the sum of ELISpot responses towards the serotypes 2, 6A, 9N, and 14 using the optimized ELISpot conditions (n = 25). We observed positive correlation (r = 0.32, p = 0.12), as shown in Figure 5. We also considered the individual ELISpot assays and observed positive correlation in all four serotypes (PS 2: r = 0.04; PS 6A: r = 0.15; PS 9N: r = 0.28; PS 14: r = 0.36). Moreover, data on PS 11A were available in 22 out of 25 patients (r = 0.26).

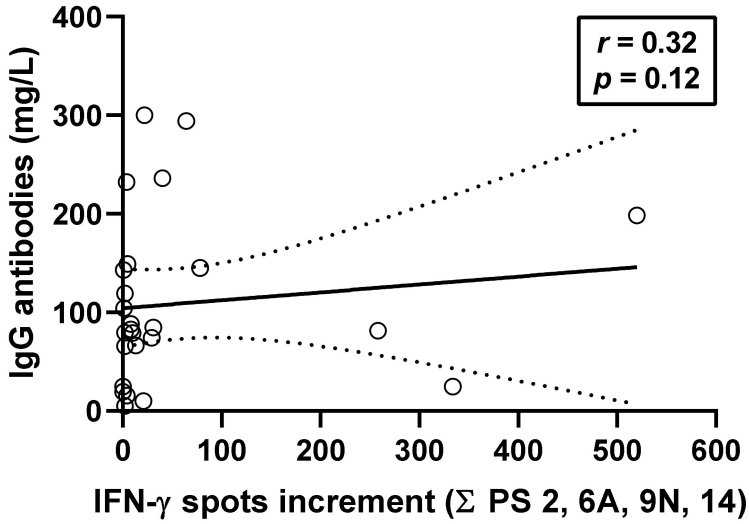


Figure 5. Spearman correlation analysis of ELISpot results and IgG antibodies against *S. pneumoniae*. This analysis considers results of kidney transplant recipients at month 12, i.e., after having received vaccination with Prevenar 13 and Pneumovax 23. We summed up ELISpot responses towards the pneumococcal serotypes (PS) 2, 6A, 9N, and 14, tested at concentrations of 100, 150, and 200 µg/mL. We used median values for the three concentrations of each serotype, yielding a single result per sample. IgG antibodies against 23 pneumococcal serotypes were determined in parallel by commercial ELISA (n = 25). The continuous line represents the regression line and the broken lines the 95% confidence interval.

3.5. Correlation between Pneumococcus-Specific ELISpot Responses and Patient Characteristics

Spearman analysis indicated that ELISpot responses at month 12 correlated in five out of six serotypes positively with the interval between transplantation and vaccination, reaching statistical significance for the serotype PS 6A (r = 0.37, p = 0.04). Thus, patients vaccinated later after transplantation had higher cellular responses. Males displayed on average 1.1-fold higher responses than females, which was non-significant. Age had no definite effect on cellular responses (r = −0.35—r = 0.25). As 25 out of 33 patients tested at month 12 received a tacrolimus-based immunosuppressive regimen, the impact of immunosuppressive drugs could not be adequately analyzed in our rather small cohort.

4. Discussion

In our current study, we describe the establishment of an IFN-γ ELISpot assay to detect specific immunity against *S. pneumoniae* in vaccinated kidney transplant recipients. Compared to a previous study on vaccinated healthy individuals, ELISpot responses to pneumococcal polysaccharides appeared to be overall lower in the transplant patients [17]. However, there were major differences in the experimental setting. Whereas the previous study by Wuorimaa et al. used the complete polysaccharide vaccine (without adjuvants) as antigen, we here used single polysaccharide serotypes. Thereby, we could measure serotype-specific responses. Other prior studies on cellular immunity after pneumococcal vaccination used the conjugate, diphtheria toxin [18], or the complete vaccine containing the toxin [17,19,20] as antigenic stimulus. Thereby, the cellular assays were not specific for pneumococci but could also show responses to the conjugate. Furthermore, there are some studies on cellular immunity, using either bacterial lysates, supernatants, or pneumococcal surface protein A, a cell wall-associated surface protein of *S. pneumoniae*, irrespective of vaccination and irrespective of transplantation [21–24]. These previous reports indicate that CD4+ T cell responses against *S. pneumoniae* are measurable by IFN-γ ELISpot or flow cytometry, detecting IFN-γ or IL-17 production and CD154 or CD25 expression, respectively. Using PBMC of healthy adults, Wuorimaa et al. showed that pneumococcus-specific IFN-γ secretion was prominent and increased after the vac-

cination with protein-conjugated and non-conjugated pneumococcal vaccines [17]. Of note, in volunteers receiving non-conjugated pneumococcal vaccines, IFN-γ responses towards polysaccharide antigens (without protein carrier) increased after vaccination (pre-vaccination: 0, day 14: 32, day 28: 22; data represent mean numbers of IFN-γ secreting cells). Thus, our current findings are in line with that previous study.

It needs to be clarified which cell type is the source of IFN-γ secretion. According to the spot characteristics (large and intense), the IFN-γ-producing cells could be T cells. When an experimental cellular mouse model was used, clonotype mapping of in vivo and in vitro pneumococcal polysaccharide-activated CD4+ T cells revealed clonotypic T cell receptor (TCR) transcripts [25]. It was suggested that zwitterionic polysaccharides induced oligoclonal CD4+ T cell activation, which was dependent on antigen-presenting cells [25]. The authors proposed that polysaccharides can bind to the outer part of the MHC class II binding groove and that they were recognized by the CDR3 binding domain of the TCR. Presentation by MHC class II molecules also required the presence HLA-DM molecules. However, it has been shown by Avci et al. that CD4+ T clones only recognize and react with carbohydrate in MHC class II context when presented by a peptide [9]. But we used polysaccharides without a protein carrier as stimuli. One could also speculate that the responding cells may not be αβ but γδ T cells or NKT cells. γδ T cells do not seem to require antigen processing and MHC presentation of peptide epitopes [26]. They are a minor population in the peripheral blood, bridge between the innate and the adaptive immune system, and use their TCR as a pattern recognition receptor [26]. Moreover, there has been evidence for the involvement of lung-specific γδ T cell subsets in local responses to *S. pneumoniae* infection [27]. Finally, the source of IFN-γ could be NK cells, as described previously after stimulation with lipopolysaccharide, a major component of the outer membrane of Gram-negative bacteria [28]. Of note, Kanevskiy et al. stimulated the NK cells by lipopolysaccharides, but not by pneumococcal polysaccharides (from the Gram-positive bacterium *S. pneumoniae*).

Whereas several studies on humoral immunity in transplant recipients indicate that these patients can mount an antibody response after vaccination—although at a reduced level—[12–16], data on specific cellular immunity after vaccination are not yet published in this cohort. It was a challenge to establish an assay detecting cellular immune responses against *S. pneumoniae* in kidney transplant recipients, as it is even at a low level in healthy controls [17]. It had not yet been defined whether these patients could develop a cellular reaction against pneumococci despite lifelong immunosuppressive treatment. We here report that kidney transplant recipients displayed an increase of cellular pneumococcal immunity after vaccination. PS 9N and 14—both components of Pneumovax 23—induced overall the strongest cellular immune response. The finding fits well with humoral data in vaccinated kidney transplant recipients [29]. This previous study showed a strong increase of antibodies directed against the two serotypes 9N and 14 after vaccination with Pneumovax 23. Moreover, antibodies directed against serotype 14 vs. 12 other serotypes reached the highest concentration in pooled serum of 278 healthy volunteers immunized with Pneumovax 23 [30], indicating that this serotype is highly immunogenic. Similar to antibody responses, there is great variation between individuals, most likely due to prior infection with *S. pneumoniae* and variable degree of immunosuppression. Currently, there is no gold standard to detect cellular responses against *S. pneumoniae*, and it is thus difficult to determine sensitivity and specificity of our ELISpot assay. Furthermore, the analysis is complicated by the fact that frequent natural infection with pneumococci could also lead to humoral and cellular immune responses.

As we observed a dose dependency of ELISpot responses (with a bell-shaped dose-response curve) and an increase of responses after vaccination, the results fulfil major characteristics of antigen-specific cellular responses. Whereas we could clearly detect serotype specific IFN-γ production by the ELISpot method, proliferative responses were overall at a low level. This finding fits well with the previous study on vaccinated healthy

controls, which could also not detect specific T-cell proliferation after stimulation with the polysaccharide vaccine [17].

As shown by Spearman analysis, cellular and humoral immunity against *S. pneumoniae* correlated positive but rather weakly (r = 0.32). This finding is in line with immune responses against other microbial antigens, e.g., against hepatitis B virus (r = 0.38) [31] or SARS-CoV-2 virus (r = 0.20–0.52, depending on the antigenic stimulus and the cohort) [32].

We previously analyzed the impact of immunosuppressive treatment on serotype-specific humoral immunity after vaccination with Prevenar 13 [15]. Our study showed that 35 kidney transplant recipients with vs. 14 without mycophenolate mofetil treatment responded to vaccination with less increase in opsonophagocytic killing assay (OPA) titers as well as global and serotype-specific anti-pneumococcal capsular polysaccharide (PCP) IgG, IgG2, and IgA at months 1 and 12 post-vaccination. Thirty-three patients receiving tacrolimus had higher OPA titers and serotype-specific anti-PCP IgG compared to 16 who did not. Furthermore, they displayed higher IgA concentrations 12 months after vaccination. Taking the positive correlation between cellular and humoral immunity into account, one could speculate that also cellular immunity against pneumococci is dependent on the immunosuppressive regimen.

Although measurement is recommended, the predictive value of antibody titers against *S. pneumoniae* with regard to infection protection remains unclear in transplant patients. Hopefully, T-cell response as detected by the ELISpot method does correlate better with the occurrence of clinical events. Nevertheless, this needs to be analyzed.

In conclusion, the ELISpot method we describe in the current paper will allow for further studies. These could help to define factors influencing specific cellular immunity against pneumococci in a transplant cohort or the duration of cellular immunity after vaccination.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Essen, Germany (14-5858-BO).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Cellular and Humoral Immunity against Different SARS-CoV-2 Variants Is Detectable but Reduced in Vaccinated Kidney Transplant Patients

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Abstract: In kidney transplant (KTX) patients, immune responses after booster vaccination against SARS-CoV-2 are inadequately examined. We analyzed these patients a median of four months after a third/fourth vaccination and compared them to healthy controls. Cellular responses were analyzed by interferon-gamma (IFN-γ) and interleukin-2 (IL-2) ELISpot assays. Neutralizing antibody titers were assessed against SARS-CoV-2 D614G (wild type) and the variants alpha, delta, and omicron by a cell culture-based neutralization assay. Humoral immunity was also determined by a competitive fluorescence assay, using 11 different variants of SARS-CoV-2. Antibody ratios were measured by ELISA. KTX patients showed significantly lower SARS-CoV-2-specific IFN-γ responses after booster vaccination than healthy controls. However, SARS-CoV-2-specific IL-2 responses were comparable to the T cell responses of healthy controls. Cell culture-based neutralizing antibody titers were 1.3-fold higher in healthy controls for D614G, alpha, and delta, and 7.8-fold higher for omicron ($p < 0.01$). Healthy controls had approximately 2-fold higher concentrations of potential neutralizing antibodies against all 11 variants than KTX patients. However, more than 60% of the KTX patients displayed antibodies to variants of SARS-CoV-2. Thus, KTX patients should be partly protected, due to neutralizing antibodies to variants of SARS-CoV-2 or by cross-reactive T cells, especially those producing IL-2.

Keywords: ELISpot; T cells; vaccination

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1. Introduction

Since the first appearance of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) in December 2019, more than 500 million people have been infected with SARS-CoV-2 and more than 6 million people have died from coronavirus disease 19 (COVID-19) (June 2022) [1].

Immunocompromised individuals, such as cancer patients, solid organ recipients, and individuals with comorbidities, have a higher mortality and morbidity rate from COVID-19 [2–4]. Individuals who belong to vulnerable groups benefit from vaccination against

SARS-CoV-2 to protect themselves from infection. They can also be protected indirectly by vaccinating individuals around them, as this significantly reduces the risk of infection [5–7].

However, studies displayed only weak or no vaccination responses after SARS-CoV-2 infection and two mRNA vaccinations in immunosuppressed patients who also suffer more frequently from vaccine breakthrough infection [8–10]. Previous studies have shown that multiple vaccinations against SARS-CoV-2 can lead to an increase in the immune response of immunocompromised individuals [8,10–12]. So far, there is insufficient data on whether booster vaccination leads to adequate immune responses, especially with regard to the currently predominant SARS-CoV-2 variants delta and omicron.

In the present study, we focused on cellular and humoral immunity to SARS-CoV-2 and its variants in immunosuppressed and immunocompetent vaccinated individuals after at least three mRNA vaccinations. We analyzed cellular immunity by a fluorescence ELISpot assay, which can detect the secretion of IFN-γ and IL-2 simultaneously, as well as by colorimetric SARS-CoV-2-specific IFN-γ and IL-2 ELISpot assays. Neutralizing antibody titers to SARS-CoV-2 D614G (wild type) and its alpha, delta, and omicron variants were analyzed by a cell culture-based neutralization assay. Moreover, potential neutralizing antibodies to variants and mutants of SARS-CoV-2 were determined by competitive fluorescence assay. SARS-CoV-2-specific IgG antibodies were measured by semiquantitative ELISA.

2. Materials and Methods

2.1. Volunteers

The patient cohort comprised 32 kidney transplant (KTX) patients after booster vaccination against SARS-CoV-2 (Table 1) and without SARS-CoV-2 infection at the timepoint of blood collection. Kidney transplantation was performed at a median of 2 years (range 0.4–11.8) before blood collection. The group included 12 males and 20 females with a median age of 54 years (range 21–76). Of the 32 KTX patients, 31 were vaccinated with Comirnaty® (BioNTech/Pfizer, Mainz, Germany) and one with Spikevax® (Moderna, Cambridge, Massachusetts). Twenty-four of the KTX patients were triple-vaccinated and eight were quadruple-vaccinated. The booster vaccination took place a median of 111 days (range 43–212) before testing. The majority of patients received an immunosuppressive regimen consisting of tacrolimus, mycophenolate, and prednisone. Immunosuppressive therapy was also provided at the time of blood collection and beyond.

Table 1. Overview of the study cohort.

Characteristics [1]	Kidney Transplant Recipients	Healthy Controls
sex	12 males 20 females	5 males 12 females
age, y	54 (21–76)	53 (35–65)
tacrolimus	32 (100%)	Ø
mycophenolate	26 (81%)	Ø
belatacept	2 (6%)	Ø
prednisone	32 (100%)	Ø
interval kidney transplantation—blood collection	2 years (0.4–11.8)	Ø
interval vaccination—blood collection	111 days (43–212)	182 days (69–213)

[1] The data indicate either the median (range) or absolute numbers (percentage). The characteristics of both groups did not differ significantly, as analyzed by Fisher's exact test (sex: $p = 0.8$) or Mann–Whitney test (age: $p = 0.5$; interval vaccination—blood collection: $p = 0.1$), respectively. Ø: no medication/ no data available.

We included 17 healthy volunteers after the third vaccination without SARS-CoV-2 infection prior to blood collection as a control group. Of the 17 healthy volunteers, 11 were vaccinated with Spikevax® (Moderna, Cambridge, MA, USA) and six were vaccinated with Comirnaty® (BioNTech/Pfizer, New York, NY, USA). The group consisted of 5 males and

12 females and the median age was 53 years (range 35–65). The cohort was tested at a median of 182 days (range 69–213) after the third vaccination.

This study was approved by the ethics committee of the University Hospital Essen, Germany (20-9753-BO), and all volunteers provided informed consent to participate. It has been performed in accordance with the ethical standards noted in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

2.2. CoV-iSpot for Interferon-γ and Interleukin-2

In 31 samples (21 KTX patients, 10 healthy controls), we simultaneously stained for IFN-γ and IL-2 using the CE-marked CoV-iSpot (AID, Strassberg, Germany), as previously described [13]. This fluorescence ELISpot (Fluorospot) contains a peptide mix of the wild type SARS-CoV-2 spike protein. Duplicates of 200,000 peripheral blood mononuclear cells (PBMC) were grown with or without adding the peptide mix (S-pool). The cut-off definition was described previously [14]. We chose 5 as cut-off for positivity for IFN-γ and for IL-2. Among the positive controls, we found an average of 410 spots (range 50–880) in KTX patients for IFN-γ and 463 spots (range 50–1100) for IL-2. In the healthy controls, we found an average of 679 spots (range 486–904) for IFN-γ and 545 spots (range 422–660) for IL-2 in the positive controls.

2.3. In-House ELISpot Assay

To further analyze SARS-CoV-2-specific cellular immunity, we used IFN-γ and IL-2 ELISpot assays separately, as previously described [13]. Briefly, 250,000 PBMC of 32 KTX patients and 17 healthy controls were cultured in the presence or absence of either PepTivator® SARS-CoV-2 wild type protein S1/S2, protein S1 (600 pmol/mL of each peptide, Miltenyi Biotec, Bergisch Gladbach, Germany), of the wild type protein S1 (4 µg/mL, Sino Biological, Wayne, PA, USA.) or the omicron variant of the protein S1 (SARS-CoV-2 B.1.1.529, 4 µg/mL, Sino Biological) in 150 µL of AIM-V®. Spot numbers were analyzed by an ELISpot reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). The average values of duplicate cell cultures were included. SARS-CoV-2-specific spots were determined as the stimulated minus non-stimulated values (spots increment). We chose a spot increment of 3 for positivity for IFN-γ as well as for IL-2. In the positive controls, we saw on average 432 spots (range 200–600) in KTX patients and 464 spots (range 250–600) in healthy controls for IFN-γ. For IL-2, we saw on average 508 spots (range 200–600) in KTX patients and 517 spots (range 400–600) in healthy controls.

2.4. Cells and Viruses

A549-AT cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 4 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO_2. The clinical SARS-CoV-2 isolates D614G (wild type), alpha, delta, and omicron were obtained from nasopharyngeal swabs of COVID-19 patients at our hospital. The SARS-CoV-2 spike gene was sequenced and the corresponding variants were determined after sequence analysis with the WHO list of variants of concern [15]. The viruses were propagated on A549-AT cells and stored at −80 °C. Viral titers were determined using a standard endpoint dilution assay and calculated as 50% tissue culture infective dose (TCID50)/mL, as previously described [16].

2.5. Assessment of Neutralizing Antibodies by Cell Culture-Based Neutralization Assay

To assess the neutralizing antibody titers of sera from 28 KTX patients and 11 healthy controls, we used a standard endpoint dilution assay, as described previously [13,17,18]. From the respective sera, serial dilutions (1:20 to 1:2560) were incubated with 100 $TCID_{50}$ of SARS-CoV-2 D614G (wild type), alpha (B.1.1.7), delta (B.1.617.2) or omicron (BA.1) for one hour at 37 °C. Thereafter, the dilutions were added to confluent A549-AT cells [18] in 96-well microtiter plates. After three days of incubation, cells were stained with crystal violet (Roth, Karlsruhe, Germany) solved in 20% methanol (Merck, Darmstadt, Germany).

Cells were evaluated for the presence of cytopathic effects (CPE) by light microscopy. The neutralizing titer was defined as the reciprocal of the highest serum dilution at which no CPE was observed in any of the three test wells. A549-AT cells overexpress carboxypeptidase angiotensin-I-converting enzyme 2 (ACE2) receptor and the cellular transmembrane protease serine 2 (TMPRSS2), enabling enhancement of CPE and high SARS-CoV-2 susceptibility. A549-AT cells were cultivated in minimum essential media (MEM), supplemented with 10% (*v/v*) FCS, penicillin (100 IU/mL), and streptomycin (100 µg/mL) at 37 °C in an atmosphere of 5% CO_2 (all Life Technologies Gibco, Darmstadt, Germany).

2.6. Assessment of Neutralizing Antibodies by Competitive Immunofluorescence

For the detection of potential neutralizing antibodies against wild type SARS-CoV-2 and 11 variants of SARS-CoV-2, we used a commercial competitive immunofluorescence assay (Bio-Plex Human SARS-CoV-2 Variant Neutralization Antibody 11-Plex Panel, BIO-RAD, Hercules, CA, USA), as described previously [13]. This competitive immunofluorescence assay works like a binding inhibition assay. Magnetic beats covered with different SARS-CoV-2 spike variants are incubated with soluble, biotin-conjugated ACE2 receptors in the presence of patient sera. Neutralizing serum antibodies compete for binding to the immobilized spike proteins with biotinylated ACE2 receptors. Detection of bound ACE2 receptors is achieved by the addition of streptavidin–phycoerythrin (SA-PE), which binds to the biotinylated ACE2 receptor. The benefit of this method is to detect antibodies that can bind to different mutants and variants of SARS-CoV-2. The upper limit of the system is 1000 ng/mL. We chose 175 ng/mL as the cut-off for positivity, which was defined for a similar testing system [19].

2.7. Antibody ELISA

SARS-CoV-2-specific antibodies were detected by a CE-marked Anti-SARS-CoV-2 IgG semiquantitative ELISA (Euroimmun, Lübeck, Germany), according to the manufacturer's instructions, as described previously [14]. The ELISA plates were coated with wild type recombinant SARS-CoV-2 spike protein (S1 domain). Serum samples were analyzed automatically at a dilution of 1:100, using the Immunomat (Virion\Serion, Würzburg, Germany). An antibody ratio >1.1 was considered positive, ≥0.8 to <1.1 borderline, and <0.8 negative.

2.8. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9.4.0 (San Diego, CA, USA) software. We used Mann–Whitney tests and Spearman correlation to analyze the numerical variables. To compare the categorical variables, we used Fisher's exact test. Two-sided p values < 0.05 were considered significant.

3. Results

3.1. T Cell Responses in Kidney Transplant Patients and Healthy Volunteers

We examined the cellular immune response in KTX patients and healthy volunteers after booster vaccination and detected significant differences in the commercial CoV-iSpot upon stimulation with the S pool of wild type SARS-CoV-2 (Figure 1). Of the 21 KTX patients, six showed a positive response for IFN-γ, and seven showed a positive response for IL-2. There was a positive reaction only in one KTX patient in the ELISpot measuring simultaneous secretion of IFN-γ and IL-2. Of the 11 healthy controls, seven showed a positive reaction for IFN-γ, seven for IL-2, and two for the simultaneous secretion of IFN-γ and IL-2. The spot increment for IFN-γ and the simultaneous secretion of IFN-γ and IL-2 differed significantly between KTX patients and healthy volunteers (IFN-γ: $p = 0.005$; IFN-γ and IL-2: $p = 0.001$).

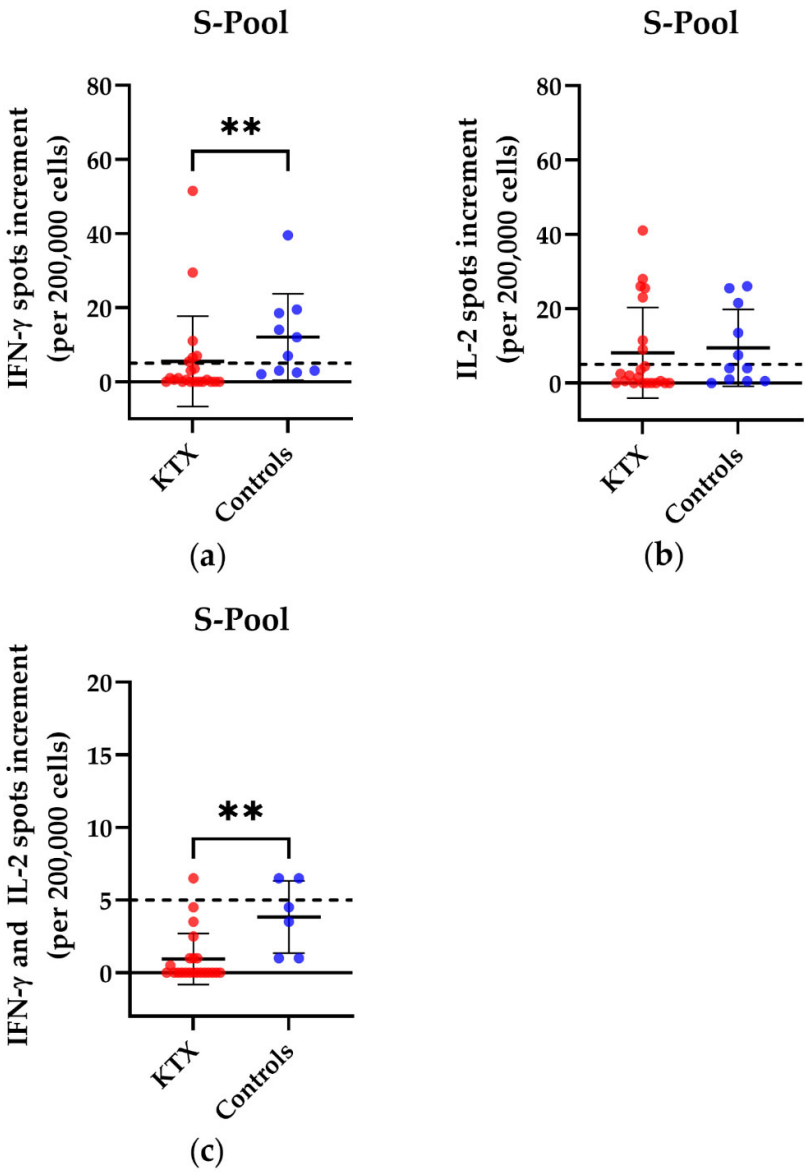

Figure 1. SARS-CoV-2-specific CoV-iSpot responses in kidney transplant (KTX) patients and healthy volunteers after booster vaccination. Distribution of (**a**) IFN-γ, (**b**) IL-2, and (**c**) simultaneous IFN-γ and IL-2 CoV-iSpot responses after stimulation with the S pool of the wild type SARS-CoV-2. Please note the different scales. Red circles show data of the KTX patients, while blue circles indicate data of the healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (** $p < 0.01$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars. The horizontal line shows the zero line. The dashed line indicates the cut-off.

Using our in-house ELISpot, we observed in KTX patients versus healthy controls significantly lower numbers of IFN-γ spots after stimulation with S1/S2, S1 or with a recombinantly expressed S1 protein (called S1 Sino hereinafter) (S1/S2: $p < 0.0001$; S1: $p < 0.0001$; S1 Sino: $p = 0.0005$) (Figure 2a,c,f). We also detected significantly lower numbers of IFN-γ spots after stimulation with a recombinant S1 protein of the omicron (B 1.1.529) variant ($p = 0.0005$) (Figure 2g). For IL-2, we could not observe significant differences between KTX patients and healthy volunteers. For IFN-γ, six of the 32 patients displayed a positive reaction towards the S1/S2 peptide mix, seven towards the S1 peptide mix, five to the S1 Sino, and five to the recombinant S1 protein of the omicron variant. For IL-2, 11 of the 32 KTX patients displayed a positive reaction towards S1/S2, 12 towards S1, 13 towards

S1 Sino, and 12 to the recombinant S1 protein of the omicron variant. Of the 17 healthy controls, 12 exhibited a positive response to the S1/S2 peptide mix, 15 to the S1 peptide mix, ten to the S1 Sino, and 11 to the recombinant S1 protein of the omicron variant. For IL-2, 11 of the 17 healthy volunteers showed a positive reaction towards S1/S2, 10 towards S1, 5 towards S1 Sino, and 9 to the recombinant S1 protein of the omicron variant. We could not detect significant differences in the cellular immune response between KTX patients after the third vaccination and KTX patients after the fourth vaccination.

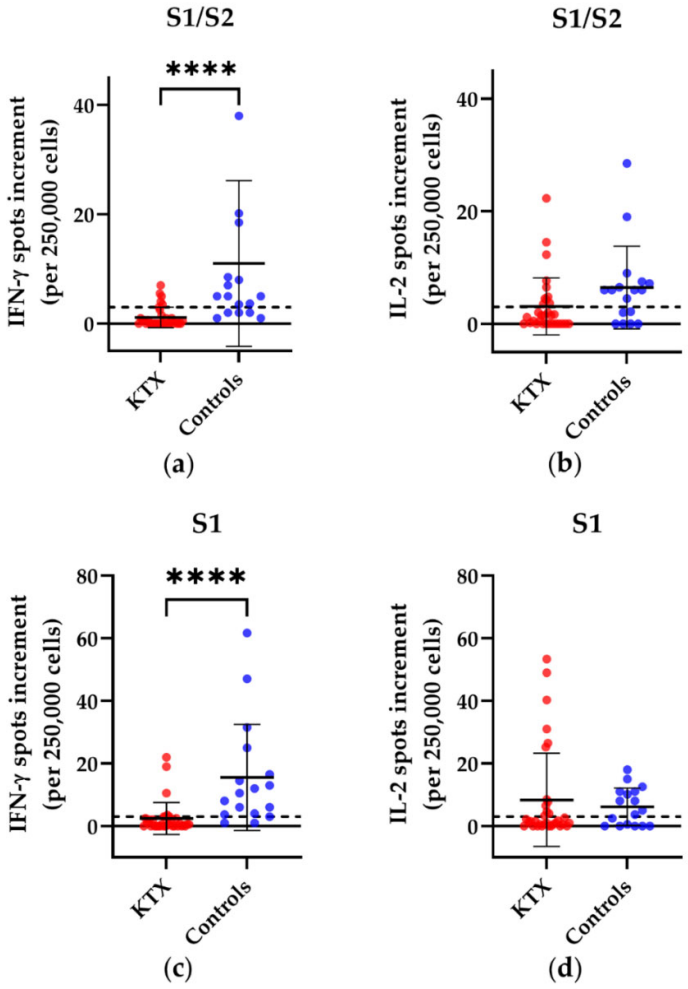

Figure 2. *Cont.*

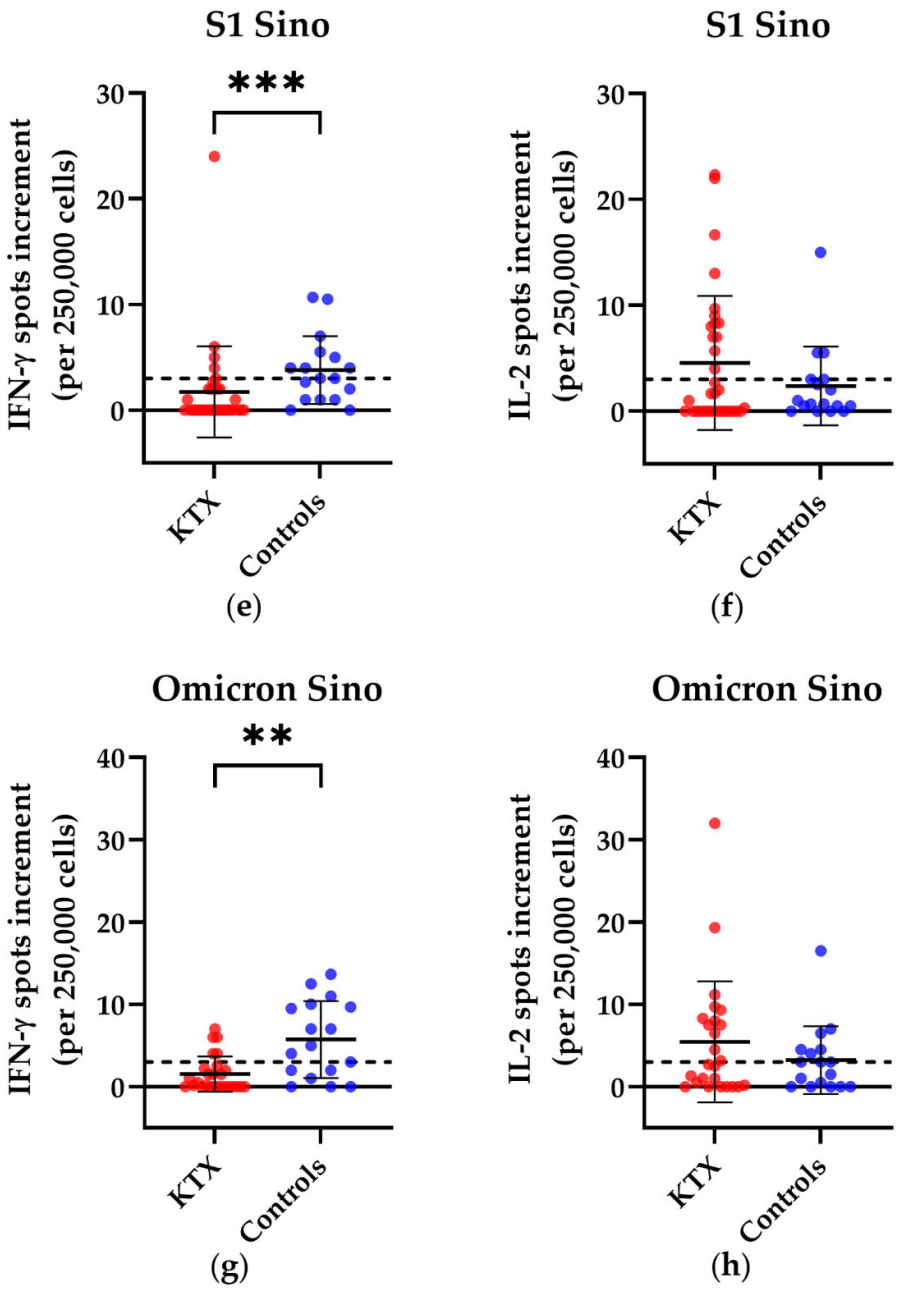

Figure 2. SARS-CoV-2-specific responses in kidney transplant (KTX) patients and healthy controls after booster vaccination, using our in-house ELISpot assay. Distribution of (**a**) IFN-γ and (**b**) IL-2 ELISpot responses after stimulation with an S1/S2 peptide mix, with an S1 peptide mix (**c**,**d**), S1 Sino (**e**,**f**) and S1 Sino of the omicron variant (**g**,**h**). Red circles show data of the KTX patients, while blue circles indicate data of the healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars. The horizontal line shows the zero line. The dashed line indicates the cut-off.

Summarizing the cellular data, KTX patients showed significantly lower SARS-CoV-2-specific responses for IFN-γ, but similar mean values for IL-2, compared to healthy controls.

3.2. Humoral Immunity in Kidney Transplant Patients and Healthy Controls

We examined the neutralizing antibodies by a cell culture-based neutralization assay and evaluated whether immunocompromised individuals could generate similar levels of neutralizing antibodies against the wild type SARS-CoV-2, alpha variant, delta variant, and

omicron (BA.1) variant as the healthy controls. KTX patients showed significantly lower titers of neutralizing antibodies than the healthy controls against all tested variants (wild type: $p = 0.0001$; alpha: $p = 0.003$; delta: $p < 0.0001$; omicron: $p = 0.0002$) (Figure 3). We could not detect significant differences between 24 KTX patients after third vaccination vs. eight KTX patients after fourth vaccination (wild type: $p = 0.7$; alpha: $p = 0.9$; delta: $p = 0.9$; omicron: $p = 0.6$).

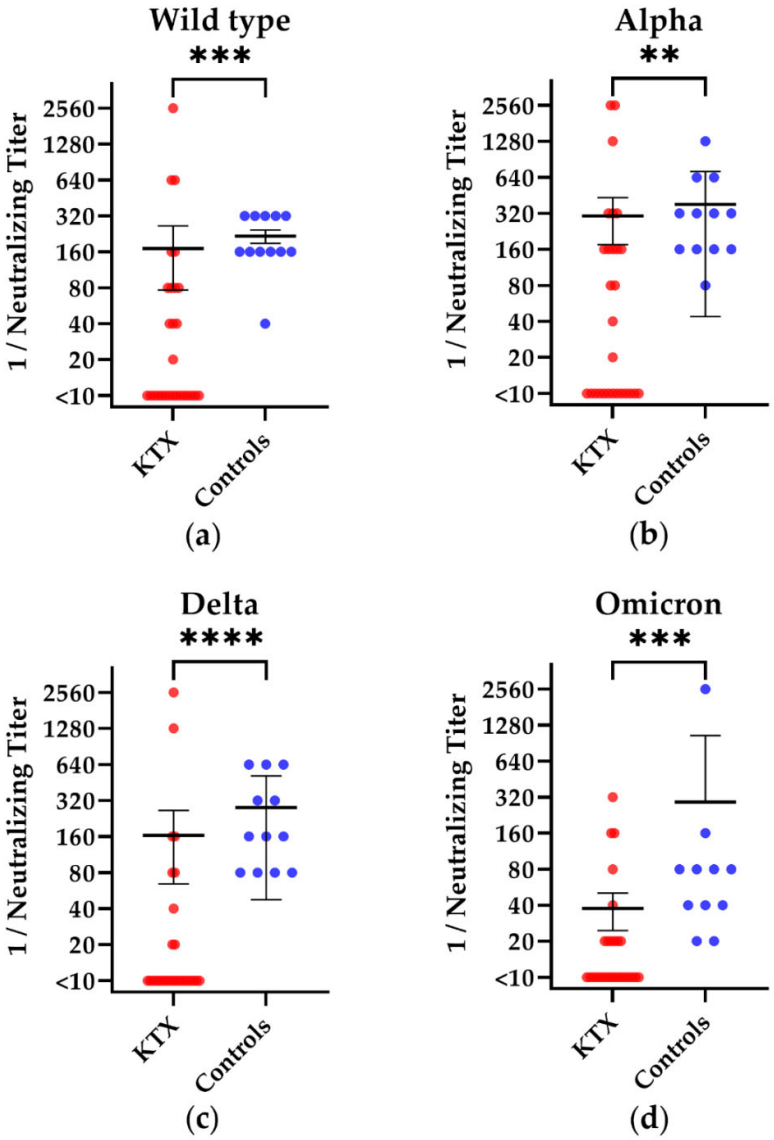

Figure 3. Titer of SARS-CoV-2-specific neutralizing antibodies in kidney transplant (KTX) patients and healthy volunteers. The reciprocal of the titer of neutralizing anti-SARS-CoV-2 (**a**)D614G (wild type), (**b**) alpha, (**c**) delta, and (**d**) omicron (BA.1) antibodies is shown on the *y*-axis. Red circles show data of the KTX patients, while blue circles indicate data of the healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars.

We also examined if vaccination can lead to a humoral immune response towards different variants and mutations of SARS-CoV-2 by a competitive immunoassay. KTX patients showed significantly lower concentrations of potential neutralizing antibodies for all tested mutations, namely, alpha, beta, gamma, delta (plus), epsilon, eta, iota, kappa, lambda, mu, and omicron (B 1.1529), compared to the healthy controls (Figure 4). In detail,

18 of the 32 patients responded to the D614G mutation, which can be found in the variants delta and omicron (Figure 4i); 22 of the 32 responded towards the K417N mutation (omicron variant, Figure 4j); and 20 of the 32 showed a positive reaction towards the N501Y mutation (omicron, Figure 4k). All 17 healthy volunteers displayed a positive response towards the D614G mutation, K417N, and N501Y. We detected significant differences between KTX patients and healthy volunteers after booster vaccination in neutralizing antibodies against all variants/mutations examined ($p < 0.001$). The comparison between 24 KTX patients after the third vaccination and eight KTX patients after the fourth vaccination did not display significant differences.

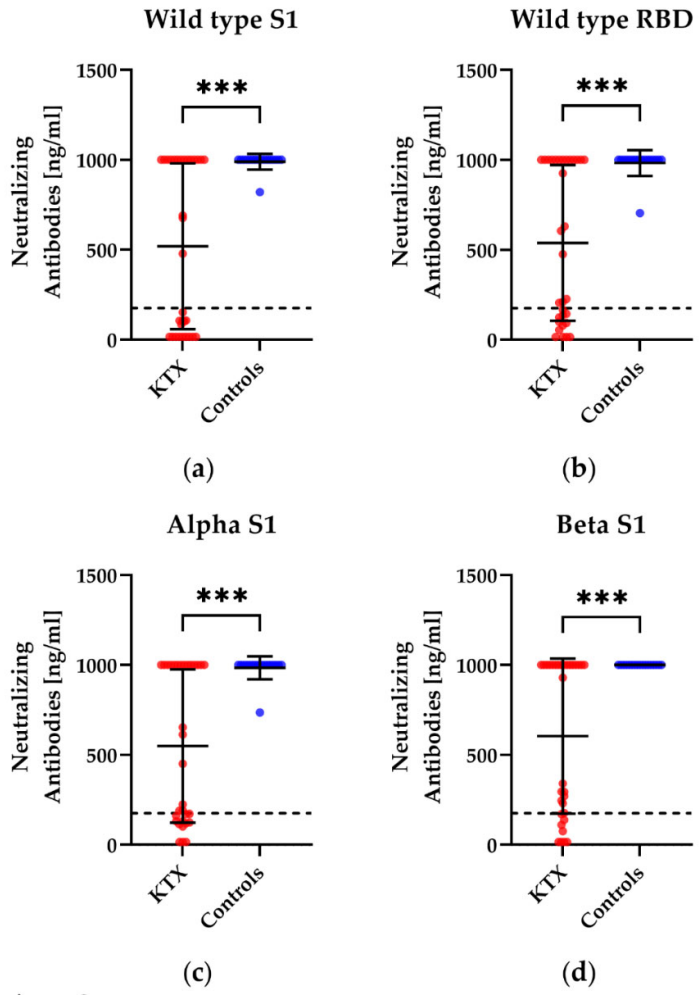

Figure 4. *Cont.*

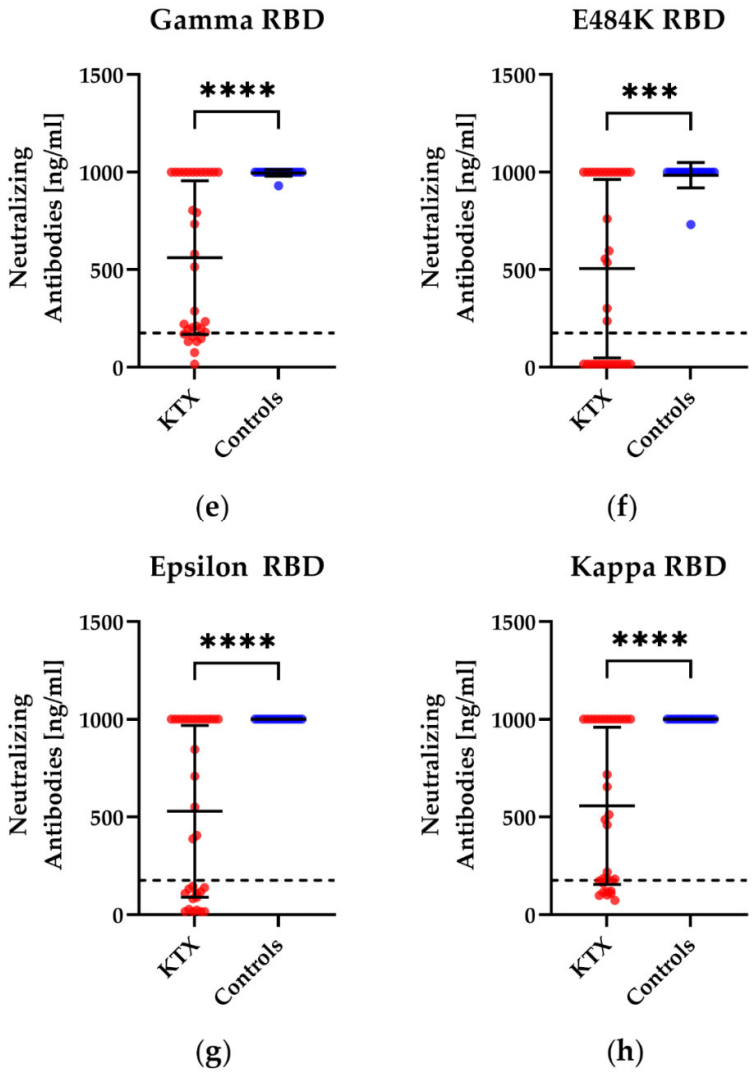

Figure 4. *Cont.*

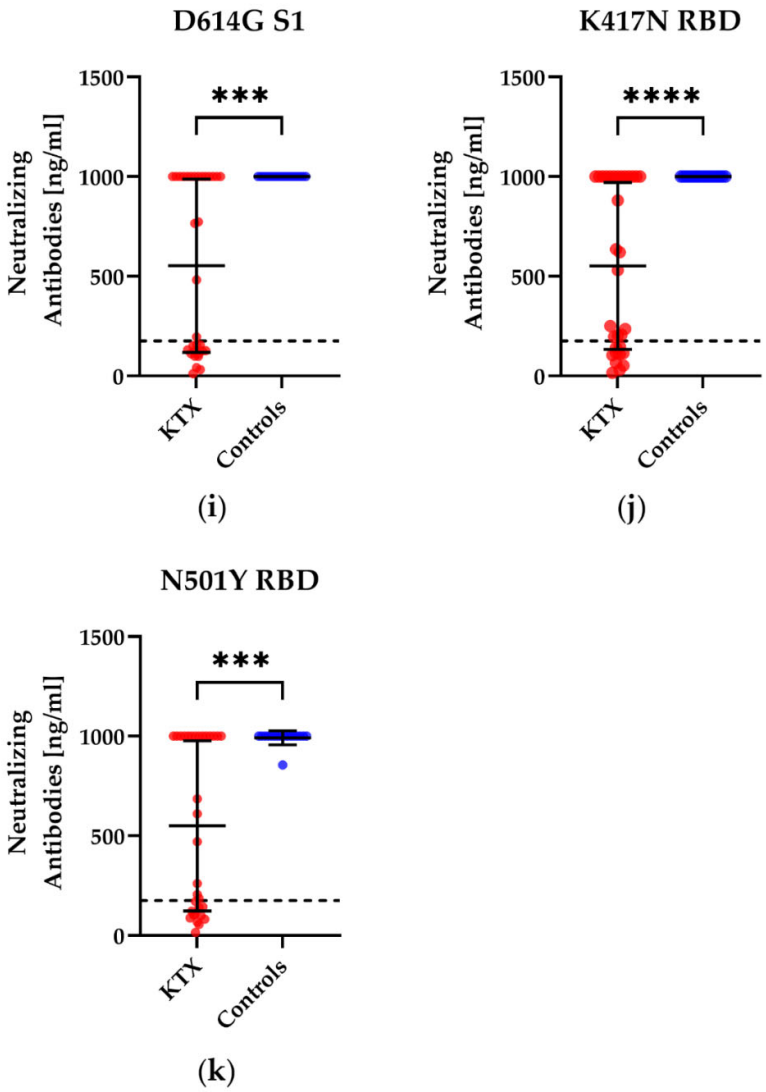

Figure 4. Concentration of potential neutralizing antibodies towards different variants of the subunit 1 of spike protein (S1) or the receptor-binding domain (RBD) of SARS-CoV-2 in kidney transplant (KTX) patients and healthy volunteers (controls) after booster vaccination. Humoral responses after booster vaccination against (**a**) wild type S1, (**b**) wild type RBD, (**c**) alpha S1, (**d**) beta S1, (**e**) gamma RBD, (**f**) E484K RBD, (**g**) epsilon RBD, (**h**) kappa RBD, (**i**) D614G S1, (**j**) K417N RBD and (**k**) N501Y RBD. The mutation D614G can be found in the delta and omicron variants, while K417N and N501Y are mutations in the omicron variant. Red circles show data of the KTX patients, while blue circles indicate the data of healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (*** $p < 0.001$, **** $p < 0.0001$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars. The dashed line indicates the cut-off.

In addition, we measured the antibody ratio in 32 KTX patients and in 17 healthy controls. We detected a significantly lower antibody ratio in KTX patients compared to healthy volunteers (mean ratio of 3.7 vs. 9.3, $p < 0.0001$) (Figure 5). We observed no significant differences between 24 KTX patients after the third vaccination and eight KTX patients after the fourth vaccination ($p = 0.7$).

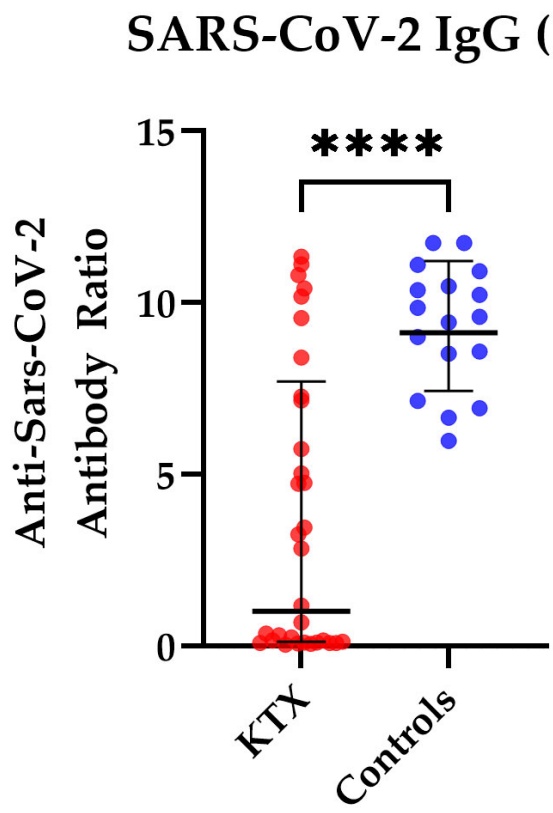

Figure 5. SARS-CoV-2-specific IgG antibody responses in kidney transplant (KTX) patients and healthy controls. SARS-CoV-2-specific IgG antibody responses are shown as antibody ratios, which determines a quotient of antibodies in the patient samples and in a control sample. Red circles show data of the KTX patients, while blue circles indicate the data of healthy volunteers. Two-tailed Mann–Whitney tests were used to compare the responses (**** $p < 0.0001$). Mean values are represented by horizontal lines, while the standard deviation is represented by error bars.

4. Discussion

We observed significant differences in cellular immunity between KTX patients and healthy controls after booster vaccination. While the lower response in the IFN-γ ELISpot was expected, comparable results in the IL-2 ELISpot were at first glance surprising. However, Schrezenmeier et al. found an increase in IL-2-secreting T cells after booster vaccination in KTX patients, whereas the IFN-γ response remained reduced [20]. This is in agreement with our results.

The results of the cell culture based neutralization assay showed comparable mean values of antibodies against the wild type, alpha, and delta, which were moderately decreased in KTX patients as compared to the healthy controls, who had a 1.3-fold higher mean value. For omicron (BA.1), however, differences between KTX patients and healthy controls were more pronounced ($p < 0.0001$). Here, the healthy controls had a 7.8-fold higher mean value of neutralizing antibody titers [21]. This could be due to the fact that these vulnerable groups are still specially protected from possible contact with the virus.

Our results demonstrate that both KTX patients and healthy controls displayed neutralizing antibodies towards variants and mutations of SARS-CoV-2 after booster vaccination against SARS-CoV-2. However, based on the detection of specific antibodies, a protective effect can hardly be assumed. Previous studies have shown that only about 40% of KTX patients develop a humoral immune response after the third vaccination [20,22]. In our study, the measured values were above the cut-off in about 64% of the KTX patients. This could indicate a better response to booster vaccination. An impact of the KTX patients after the fourth vaccination can be excluded, as they do not show any significant differences to KTX patients after the third vaccination.

We detected strongly reduced antibody ratios in KTX patients, which is consistent with the results of previous studies [20,22,23]. However, a study by Bensouna et al. observed an increase in the humoral immune response 30 days after the third vaccination. However, in our study, testing took place at a median of 111 days after vaccination. Other reasons for the lower humoral immune response could be treatment with mycophenolate mofetil or impaired germinal center immunity in immunosuppressed individuals [24].

One limitation of the present study is a lack of data on memory B cells. Notably, other studies showed impaired humoral immunity after mRNA vaccination [25]. Furthermore, it could be demonstrated that a humoral immune response is generated when immunosuppressants are paused [26]. In the cohort studied in our paper, no pausing of immunosuppressive medication was performed. Subsequent studies are needed to comprehensively analyze the memory B cell response in mRNA-vaccinated patients with immunosuppressive treatment.

Our data indicate that there is inadequate immunization in vulnerable groups when compared to healthy controls. In a previous study, we also observed an insufficient humoral immune response in HSCT patients after the third vaccination [13]. Accordingly, other studies of the humoral immune response after SARS-CoV-2 vaccination in vulnerable groups, such as organ transplant and cancer patients, also showed a reduced immune response [27–29]. For these individuals, it is recommended to follow all the related safety precautions and to monitor the humoral immune response on a regular basis.

5. Conclusions

In conclusion, cellular immunity of KTX patients was significantly lower compared to healthy controls for IFN-γ. For IL-2, KTX patients had a similar mean value of spots increment as the healthy controls. It might be possible that IL-2-secreting T cells also contribute to protection against SARS-CoV-2 infection. However, these cells are not measured by most standard tests. More than half of the KTX patients generated levels of potential neutralizing antibodies to variants of SARS-CoV-2. KTX patients developed neutralizing antibodies, even if they were significantly lower than the titers of healthy controls. Nevertheless, our data suggest that KTX patients are at least partly protected against SARS-CoV-2, either by neutralizing antibodies to variants of SARS-CoV-2 or by cross-reactive T cells.

Author Contributions: Conceptualization, M.L.; methodology, L.T., N.F., M.B., M.O. and M.A.; software, L.T.; validation, M.L. and A.G.; formal analysis, L.T. and M.L.; investigation, M.L.; resources, M.L., A.G., H.R., S.C., M.W., P.A.H. and A.K.; data curation, L.T. and M.L.; writing—original draft preparation, L.T. and M.L.; writing—review and editing, L.T., M.L., U.D., P.A.H., O.W. and A.K.; visualization, L.T.; supervision, M.L., P.A.H. and O.W.; project administration, M.L. and P.A.H.; funding acquisition, P.A.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital Essen, Germany (20-9753-BO).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

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Conflicts of Interest: The authors declare no conflict of interest.

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Prospective, Longitudinal Study on Specific Cellular Immune Responses after Vaccination with an Adjuvanted, Recombinant Zoster Vaccine in Kidney Transplant Recipients

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Abstract: Solid organ transplant recipients have an up to ninefold higher risk of varicella–zoster virus (VZV) reactivation than the general population. Due to lifelong immunosuppressive therapy, vaccination against VZV may be less effective in kidney transplant (KTX) recipients. In the current study, twelve female and 17 male KTX recipients were vaccinated twice with the adjuvanted, recombinant zoster vaccine Shingrix™, which contains the VZV glycoprotein E (gE). Cellular immunity against various VZV antigens was analyzed with interferon-gamma ELISpot. We observed the strongest vaccination-induced changes after stimulation with a gE peptide pool. One month after the second vaccination, median responses were 8.0-fold higher than the responses prior to vaccination ($p = 0.0006$) and 4.8-fold higher than responses after the first vaccination ($p = 0.0007$). After the second vaccination, we observed an at least twofold increase in ELISpot responses towards gE peptides in 22 out of 29 patients (76%). Male sex, good kidney function, early time point after transplantation, and treatment with tacrolimus or mycophenolate were correlated significantly with higher VZV-specific cellular immunity, whereas diabetes mellitus was correlated with impaired responses. Thus, our data indicate that vaccination with Shingrix™ significantly augmented cellular, VZV gE-specific immunity in KTX recipients, which was dependent on several covariates.

Keywords: varicella–zoster virus; vaccination; ELISpot; kidney transplantation; sex dependency; diabetes mellitus

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1. Introduction

Varicella–zoster virus (VZV) is a member of the herpesvirus family that causes varicella/chickenpox after primary infection and zoster/shingles after reactivation. Viral DNA persists in neurons of the dorsal root and cranial nerve ganglia, where it can remain quiescent for decades [1]. As all herpesviruses, VZV may reactivate, especially in older and immunocompromised individuals [2,3]. Waning of VZV-specific cellular immunity is an important factor for VZV reactivation, and the age-dependent increase in shingles is correlated with the decrease in specific T cell immunity [4]. The incidence of shingles was up to ninefold higher in immunosuppressed solid organ transplant recipients than in the general population [5,6]. VZV causes a vesicular exanthema affecting one to three adjoining dermatomes, where it can lead to pain and postherpetic neuralgia [1,7].

In Germany, the United States, and many other countries, a live attenuated vaccine is licensed, and its use is recommended for vaccination against primary infection [8,9]. Moreover, to prevent reactivations, the use of a recombinant, adjuvanted VZV glycoprotein

E (Shingrix™, GlaxoSmithKline Biologicals S.A., Rixensart, Belgien) is recommended, especially from the age of 60 and for individuals with immunodeficiency [8]. This recombinant zoster vaccine contains an adjuvant based on liposomes, which serves as an amplifier of immunity [1]. Previous data indicate that vaccination with Shingrix™ could reduce the risk of contracting shingles during one's lifetime in the general population from 33% to 3% [10]. Moreover, data in kidney transplant (KTX) recipients indicate that it is also effective and may cut the rate of shingles by about half [5]. Considering 130 patients who received Shingrix™ and 130 who received a placebo, a study by Vink et al. [5] reported a lower rate of suspected cases of shingles in vaccinees (3 vs. 7 suspected cases).

In the present study, we report on 29 KTX recipients who were vaccinated twice with Shingrix™, in which VZV-specific cellular immunity was monitored at four time points prior to and post vaccination. We stimulated the patient cells with peptides of glycoprotein E (gE), the most abundant and immune-dominant glycoprotein of VZV [11], with a native VZV glycoprotein and with an inactivated whole VZV antigen. Immunity against VZV (gE) was measured with a highly sensitive interferon (IFN)-γ ELISpot assay, which detects specific T cells on a single-cell level [12]. Moreover, we compared responses in the patients with healthy controls and analyzed if covariates, such as sex, age, number of kidney transplantations, kidney function, co-morbidities, prior shingles, immunosuppressive therapy, allograft rejection, and interval between transplantation and vaccination or between vaccination and testing, had an impact on VZV-specific immunity.

2. Materials and Methods

2.1. Volunteers

Our prospective single-center study includes 29 KTX recipients who were tested longitudinally before and after vaccination with Shingrix™. The participants in this observational study were recruited at the University Hospital Essen (Germany) in August 2020 according to the inclusion and exclusion criteria outlined in Table 1. The patient cohort contained twelve females and 17 males, and the median age at the time of the first blood sampling was 61 years (range: 45–79). The estimated glomerular filtration rate (eGFR, MDRD equation) [13] remained constant after vaccination (median values of 46–51 mL/min/1.73 m^2). All patients reported previous chickenpox, and eight reported shingles. Ten patients were grafted with a living donor and 19 with a deceased donor. The patients were tested at the times of the first and second vaccination and approximately one and four months after the second vaccination. The median interval between the transplantation and first vaccination was 7.2 years, and that between the two vaccinations was 71 days.

Table 1. Inclusion and exclusion criteria.

Inclusion	Exclusion
Age ≥ 45 years	Acute rejection [2]
Interval to kidney transplantation ≥ 6 months	Active shingles infection
Interval to shingles ≥ 2 months	Acute (other) infection (fever > 38.5 °C)
Stable kidney function [1]	Actual malignant tumor
Complete clinical dataset	Allergy against a component of the vaccine
Sequential ELISpot data at four time points	Pregnancy
Written informed consent	Inability to consent

[1] Estimated glomerular filtration rate of >15 mL/min/1.73 m^2 and change in serum creatinine of <1.5-fold within the month prior to inclusion; [2] defined by change in serum creatinine of >20% within one month prior to inclusion.

In parallel, in August 2020, we included four age-matched, healthy controls (median age: 62 years, range: 60–65, three males and one female). All volunteers reported previous chickenpox, and the female reported previous shingles. According to the current recommendations [8], healthy individuals should be vaccinated against shingles from the age of 60, which defined the minimum age. Of note, none of the controls received

immunosuppressive treatment. The median interval between their two vaccinations was 67 days.

The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Ethics Committee of the University Hospital Essen, Germany (19-8700-BO, 18.12.2019). Informed consent was obtained from all subjects involved in the study.

2.2. Vaccine

The subunit vaccine Shingrix™ contains 50 µg of the adjuvanted, recombinant VZV gE antigen produced in immortalized ovarian cells of the Chinese hamster (CHO cells) [14]. It is adjuvanted with AS01B containing 50 µg of the *Quillaja saponaria* Molina plant extract, fraction 21 (QS-21), and 50 µg 3-O-desacyl-4′-monophosphoryl lipid A (MPL) from *Salmonella minnesota*. Shingrix™ is licensed for the prevention of shingles and postherpetic neuralgia in adults ≥50 years of age [14]. Vaccination consisted of two 0.5 mL doses injected into the deltoid muscle.

2.3. ELISpot Assay

Nine milliliters of heparinized blood was collected, and peripheral blood mononuclear cells (PBMCs) were separated through Ficoll gradient centrifugation. Numbers of PBMCs were determined with an automated hematology analyzer (XP-300, Sysmex, Norderstett, Germany). To assess VZV-specific cellular immunity, we performed IFN-γ ELISpot assays while using a peptide pool and two protein antigens as stimuli. In parallel experiments, we applied a gE peptide pool (1 µg/mL per peptide, JPT Peptide Technologies, Berlin, Germany), a native VZV glycoprotein (10 µg/mL, SERION), and a whole native VZV antigen (10 g/mL, SERION, Würzburg, Germany). The gE peptide pool contained 153 peptides derived from a peptide scan (15-mers with 11 aa overlap) through the envelope protein (Swiss-Prot ID: P09259) of the VZV strain Dumas. For the production of the two native antigens, VZV glycoprotein, and whole VZV antigen, HEL 299 cells were infected with the VZV strain Ellen. After cultivation, the antigens were isolated through lectin affinity chromatography or ultra-centrifugation through a sucrose cushion, respectively. The production of IFN-γ was determined using pre-coated ELISpot plates and a standardized detection system (T-Track® ELISpot kit, Mikrogen GmbH, Neuried, Germany; formerly Lophius Biosciences GmbH, Regensburg, Germany). Cultures of 200,000 freshly isolated PBMCs were incubated without and with VZV antigens in 150 µL of AIMV medium (Gibco, Grand Island, USA) at 37 °C for 19 h. Stimulation with the T-cell mitogen phytohemagglutinin (PHA, 4 µg/mL) served as positive control. Colorimetric detection of cytokine-secreting cells was performed according to the manufacturer's instructions. Spot numbers were analyzed with an ELISpot plate reader (AID Fluorospot, Autoimmun Diagnostika GmbH, Strassberg, Germany). VZV-specific spots were determined as stimulated minus non-stimulated (background) values (spot increment). Of note, the negative controls reached a median value of 0, a mean of 0.11 spots, and a standard deviation of 0.61 spots. The positive control with PHA indicated that all results included in this study were valid (median: 378 spot increment, range: 46–565).

2.4. Parameters with Potential Influence on Vaccination Responses

We considered age, kidney function (eGFR), interval between transplantation and first vaccination, interval between first and second vaccination, and interval between second vaccination and blood sampling as numerical variables. Moreover, sex, first vs. second kidney transplantation, living vs. deceased donor, diabetes mellitus, hypertension, coronary heart disease, previous malignant tumor, chronic obstructive pulmonary disease, previous cytomegalovirus, herpes simplex virus or VZV infection (chickenpox or shingles), previous antiviral treatment (acyclovir, valganciclovir, entecavir, cytotect), immunosuppressive therapy (tacrolimus, mycophenolate, corticosteroids, everolimus, azathioprine, ciclosporin,

belatacept), and allograft rejections (total) were considered as categorical, dichotomous variables (yes/no).

2.5. Statistical Analysis

Data were analyzed using GraphPad Prism 8.4.2.679 (GraphPad Prism Software, San Diego, CA, USA) or IBM SPSS Statistics version 25 (Armonk, NY, USA). The calculation of the sample size was performed with the program G*Power 3.1.9.4 [15] using the following input parameters: one tail, an effect size of 0.55, an α error probability of 0.05, and a power (1-β error probability) of 0.95. This calculation yielded a total sample size of 27. The effect size was assumed based on preliminary data from a previous study [16]. Time courses of ELISpot responses were analyzed by using one-way ANOVA with Tukey's multiple-comparison test. The results in transplant patients and healthy controls were compared by using a Mann–Whitney *U*-test. Correlation analyses of numerical variables were performed by Spearman test (two-tailed). The impact of categorical variables was also analyzed with the Mann–Whitney test. The impact of clinical variables on ELISpot responses was furthermore tested with multivariate analysis (multinomial logistic regression). If not otherwise stated, median values are indicated. Results were considered significant at $p < 0.05$.

3. Results

3.1. Time Course of ELISpot Responses to Three Different VZV Antigens

In 29 KTX patients vaccinated with Shingrix™ (Table 2), we followed up the T cell responses towards a gE peptide pool, a native glycoprotein of VZV, and a whole VZV antigen (Table 3, Figure 1a–c). We observed the strongest vaccination-induced changes after stimulation with the gE peptide pool. One month after the second vaccination, median responses were 8.0-fold higher than the responses prior to vaccination ($p = 0.0006$) and 4.8-fold higher than the responses after the first vaccination ($p = 0.0007$). However, at month 4 vs. 1 after the second vaccination, ELISpot responses already declined significantly ($p = 0.01$) (Figure 1a). The results on the native glycoprotein showed a similar trend, i.e., a maximum response at month 1 after the second vaccination and, thereafter, a decrease in ELISpot responses (Figure 1b). After stimulation with the whole VZV antigen, vaccination-induced changes also reached statistical significance (Figure 1c). One month after the second vaccination, median responses were 4.1-fold higher than the responses prior to vaccination ($p = 0.03$) and 1.9-fold higher than the responses after the first vaccination ($p = 0.01$).

Table 2. Characteristics of the 29 kidney transplant recipients tested prior to and post vaccination with Shingrix™.

Variable	Group	Absolute Number or Median (Range)
Sex	Female	12
	Male	17
Age (years)		61 (45–79)
Kidney transplantation, no.	First	24
	Second	5
eGFR (mL/min/1.73 m^2)	Prior to vacc.	46 (16–94)
	Post 1st vacc.	49 (12–99)
	M1 post 2nd vacc.	51 (14–94)
	M4 post 2nd vacc.	47 (15–88)
Co-morbidities	Diabetes mellitus	4
	Hypertension	12
	Coronary heart disease	8
	Previous malignant tumor	11
	COPD	4

Table 2. *Cont.*

Variable	Group	Absolute Number or Median (Range)
Anamnesis of previous infection with herpesviruses	Cytomegalovirus	11
	Herpes simplex virus type 1	2
	VZV (chickenpox)	29
	VZV (shingles)	8
Previous antiviral treatment	Aciclovir	1
	Valganciclovir	3
	Entecavir	1
	Cytotect	1
Immunosuppressive therapy	Tacrolimus	25
	Mycophenolate	20
	Corticosteroids	26
	Everolimus	5
	Azathioprine	1
	Ciclosporin	1
	Belatacept	2
Allograft rejection	Total	6
	Acute	5
	Acute and chronic	1
	Humoral	2
	Cellular	3
	Humoral and Cellular	1
Interval transplantation–1st vaccination		7.2 years (8 months–34.7 years)
Interval 1st vaccination–2nd vaccination		71 days (62–149)
Interval 2nd vaccination–blood sampling	First follow-up	1.2 months (0.9–1.9)
	Second follow-up	4.2 months (3.7–9.6)

eGFR—estimated glomerular filtration rate; vacc.—vaccination with Shingrix™; COPD—chronic obstructive pulmonary disease; VZV—varicella–zoster virus.

Table 3. Comparison of varicella–zoster virus (VZV)-specific ELISpot responses in 29 kidney transplant (KTX) recipients and four healthy controls (HC).

Antigen	Time Point	KTX			HC			*p*
		Median	**MIN**	**MAX**	**Median**	**MIN**	**MAX**	
Glycoprotein E Peptides	Pre vacc.	**1.5**	−0.5	20.5	**10.5**	1	19	0.07
	post 1st vacc.	**2.5**	−1	22	**5.5**	2	16	0.11
	M1 post 2nd vacc.	**12**	0	60.5	**23.5**	19	66	0.09
	M4 post 2nd vacc.	**2.5**	0	53	**22**	7	85	0.04 *
Native Glycoprotein	Pre vacc.	**1.5**	0	25.5	**10**	1	42	0.10
	post 1st vacc.	**1**	0	25	**4**	2	7	0.15
	M1 post 2nd vacc.	**2**	0	18.5	**6.5**	3	17	0.09
	M4 post 2nd vacc.	**1.5**	0	38	**8**	0	24	0.17
Whole VZV Antigen	Pre vacc.	**7.5**	0	205.5	**50**	4	117	0.10
	post 1st vacc.	**16**	0	126.5	**37.5**	9	60	0.09
	M1 post 2nd vacc.	**30.5**	0	155.5	**62.5**	35	138	0.08
	M4 post 2nd vacc.	**6**	0	56.5	**40**	11	124	0.07

VZV—specific cellular immunity is indicated as the spot increment, i.e., stimulated vs. non-stimulated (background) values. Median values are highlighted in bold. MIN—minimum; MAX—maximum; M—month; vacc.—vaccination with Shingrix™. Data were compared by using a Mann–Whitney test (* $p < 0.05$).

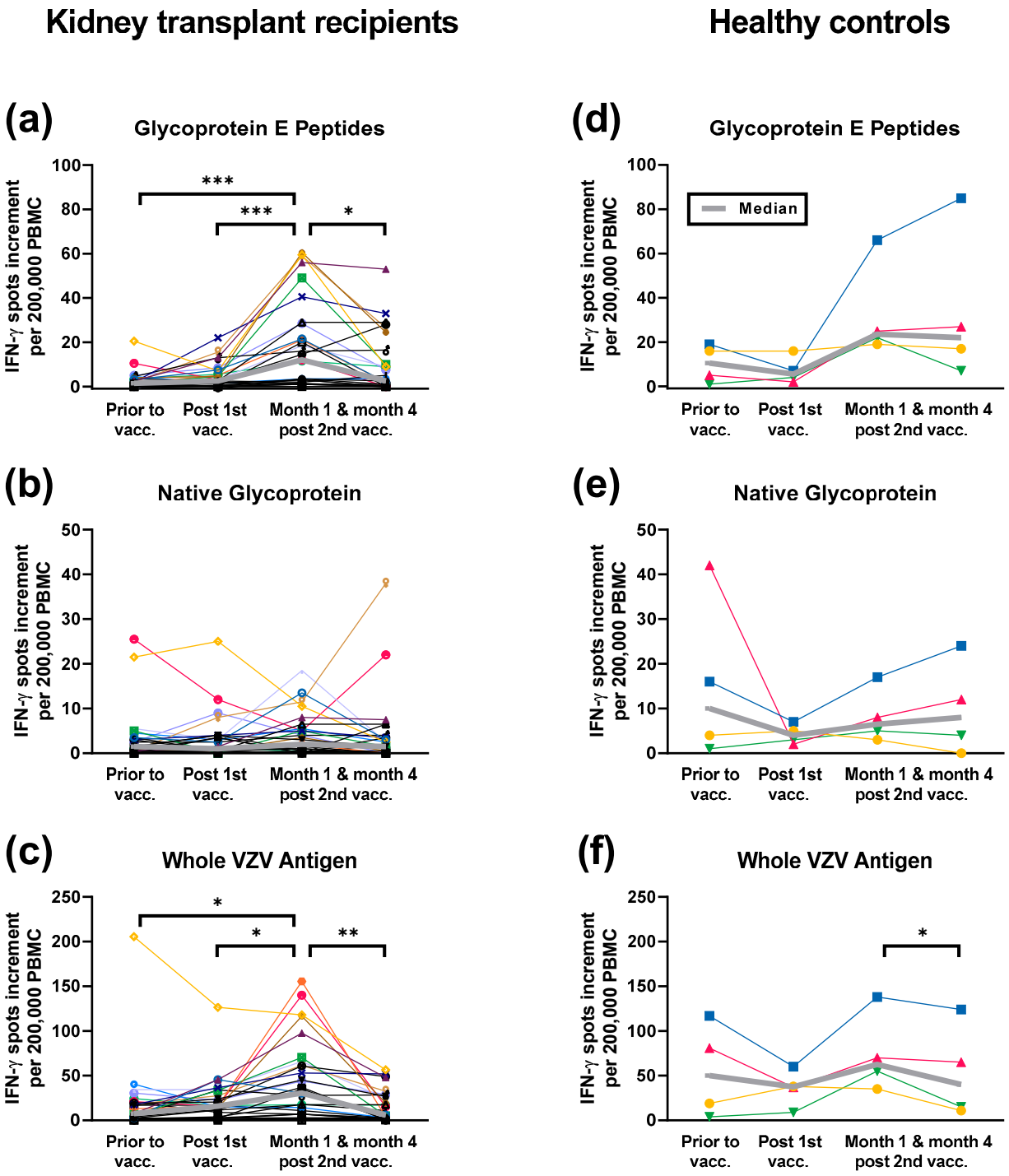

Figure 1. Time course of ELISpot responses towards various varicella–zoster virus (VZV) antigens in 29 kidney transplant recipients (**a**–**c**) and in four healthy controls (**d**–**f**). We used a peptide pool of glycoprotein E (**a**,**d**), a native glycoprotein (**b**,**e**), or a whole VZV antigen (**c**,**f**) for in vitro stimulation of peripheral blood mononuclear cells (PBMCs). Data prior to and post vaccination (vacc.) with Shingrix™ were compared by using one-way ANOVA with Tukey's multiple-comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). VZV-specific spots were determined as stimulated minus non-stimulated (background) values (spot increment). The data for each individual is coded by the same color and symbol used consistently in panels (**a**) to (**c**) or (**d**) to (**f**). The bold gray line connects the median values.

The ELISpot responses in the four vaccinated healthy controls (Figure 1d–f) were overall higher than those of the KTX recipients, reaching statistical significance ($p < 0.05$) for stimulation with the gE peptide pool at month 4 after the second vaccination (Table 3). Overall, there was a greater drop in immunity in the patients than in the healthy controls. Thus, we could detect significant VZV (gE)-specific cellular responses in vaccinated KTX recipients, and the gE peptide pool, which is the immunogenic component of the subunit vaccine Shingrix™, appeared to be the best stimulus for assessing VZV (gE)-specific cellular vaccination responses.

Moreover, we calculated how many patients showed an at least twofold increase in ELISpot responses at month 1 after the second vaccination vs. baseline. After stimulation with the gE peptide pool, 22 out of 29 patients (76%) fulfilled this criterion, which we used to assess the response rate for cell-mediated immunity. The respective number for the native glycoprotein was 6 out of 29 (21%), and for the whole VZV antigen, it was 17 out of 29 (59%).

3.2. Correlation of VZV-Specific Cellular Immunity with Clinical Parameters

With a univariate analysis, we determined if ELISpot responses were correlated with patients' characteristics, as outlined in Section 2.4. A Spearman analysis of the numerical variables indicated that the eGFR prior to vaccination was correlated positively with the ELISpot responses to the gE peptide pool ($r = 0.42$ and $p = 0.02$) and to the native glycoprotein of VZV ($r = 0.41$ and $p = 0.03$), i.e., patients with a better kidney function showed higher VZV (gE)-specific ELISpot responses at baseline (Figure 2a,b). After vaccination, however, the correlation was no longer significant.

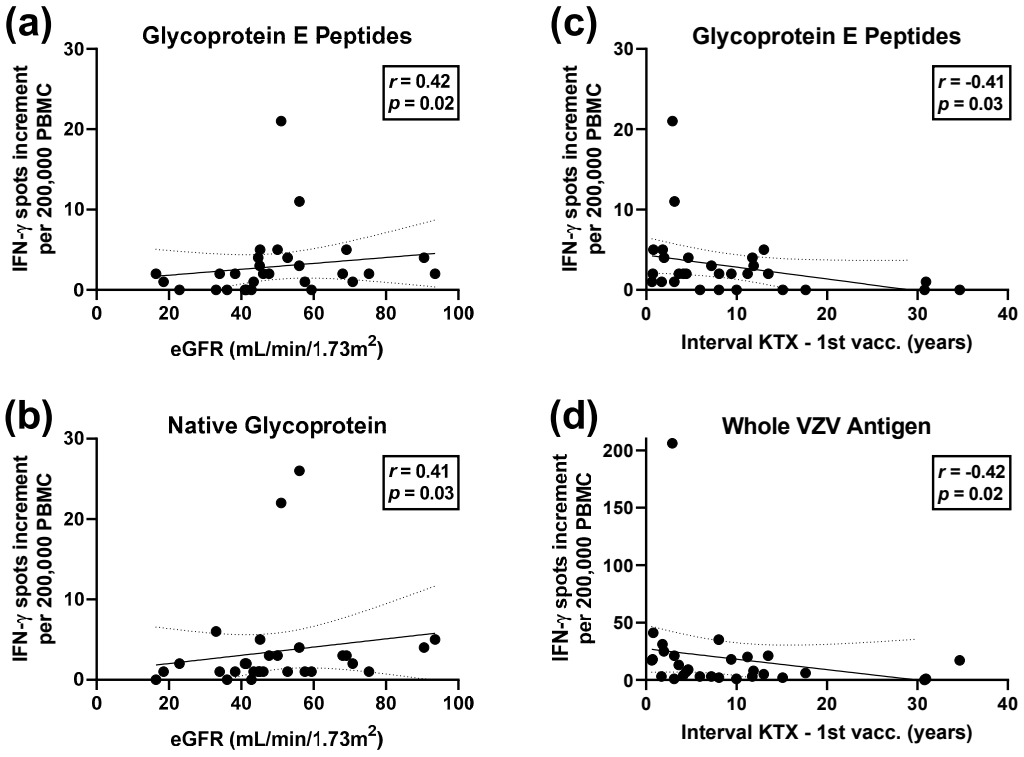

Figure 2. Spearman correlation analysis of estimated glomerular filtration rate (eGFR) or interval between transplantation and first vaccination and ELISpot responses prior to vaccination. In 29 kidney transplant recipients, we observed a positive correlation of eGFR and ELISpot responses towards a peptide pool of glycoprotein E (**a**) and towards the native glycoprotein (**b**). The correlation was negative between the interval between transplantation and first vaccination and ELISpot responses towards a peptide pool of glycoprotein E (**c**), as well as towards whole varicella–zoster virus (VZV) (**d**). The continuous line represents the regression line, and the broken lines represent the 95% confidence interval.

Moreover, the interval between transplantation and first vaccination was correlated negatively with baseline ELISpot responses to the gE peptide pool ($r = -0.41$ and $p = 0.03$) and to the whole VZV antigen ($r = -0.42$ and $p = 0.02$) (Figure 2c,d). Thus, patients tested early after transplantation showed higher VZV-specific cellular responses.

The analysis of categorical variables could identify male sex, diabetes mellitus, and treatment with tacrolimus and mycophenolate as factors influencing the cellular VZV-specific immunity. In detail, males vs. females showed stronger VZV-specific responses, which reached statistical significance for responses towards the native glycoprotein after the first vaccination ($p = 0.03$) (Figure 3). Diabetic patients had weaker cellular responses, which were significant for stimulation with the native glycoprotein prior to vaccination and at month 4 after the second vaccination ($p = 0.04$ and $p = 0.02$, respectively) (Figure 4).

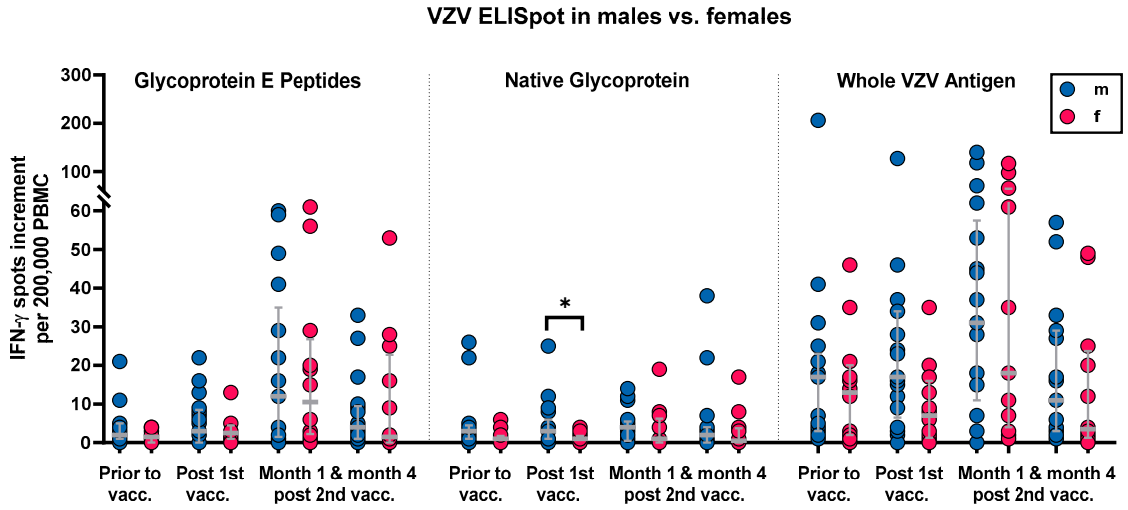

Figure 3. Varicella–zoster-virus-specific ELISpot responses in 17 male and twelve female kidney transplant recipients prior to and after the first and second vaccination with Shingrix™. Blue dots indicate males and red dots indicate females. VZV-specific spots were determined as stimulated minus non-stimulated (background) values (spot increment). Gray horizontal lines represent median values and the interquartile range. Data were compared by using a Mann–Whitney test (* $p < 0.05$).

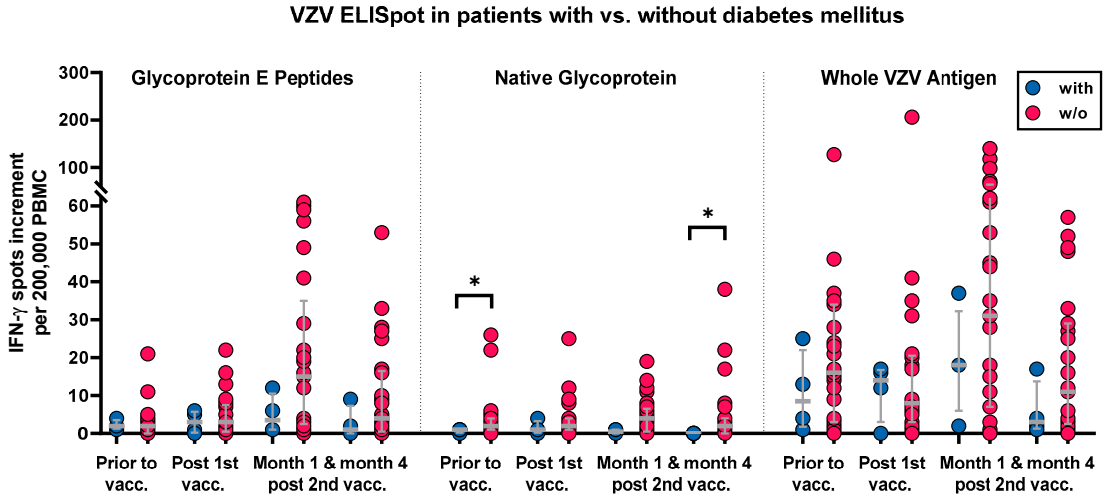

Figure 4. Varicella–zoster-virus-specific ELISpot responses in kidney transplant recipients with and without diabetes mellitus prior to and after the first and second vaccination with Shingrix™. Blue dots indicate four patients with diabetes mellitus (with) and red dots indicate 25 patients without (w/o). VZV-specific spots were determined as stimulated minus non-stimulated (background) values (spot increment). Gray horizontal lines represent median values and the interquartile range. Data were compared by using a Mann–Whitney test (* $p < 0.05$).

Patients treated with tacrolimus had stronger ELISpot responses after the second vaccination, reaching significance for the gE peptide pool at month 1 ($p = 0.02$) and for the whole VZV antigen at month 1 and month 4 ($p = 0.03$ and $p = 0.04$, respectively) (Table 4). Patients receiving mycophenolate had stronger ELISpot responses prior to vaccination and after the first and second vaccination (Table 4). The results were significant for the peptide pool, native glycoprotein, and whole VZV antigen prior to vaccination ($p = 0.03$, $p = 0.03$ and $p = 0.002$, respectively), for the whole VZV antigen after the first vaccination ($p = 0.01$), and for all three VZV antigens at month 4 after the second vaccination ($p = 0.045$, $p = 0.03$ and $p = 0.006$, respectively).

Table 4. Correlation of varicella–zoster virus (VZV)-specific ELISpot responses and immunosuppressive treatment in 29 kidney transplant recipients.

Variable	Antigen	Time Point	Treatment Received			Treatment Not Received			*p*
			Median	MIN	MAX	Median	MIN	MAX	
Tacrolimus	Glycoprotein E Peptides	Pre vacc.	**2**	0	21	**1.5**	0	2	0.32
		post 1st vacc.	**3**	0	22	**1**	0	2	0.06
		M1 post 2nd vacc.	**15**	0	61	**1.5**	0	3	**0.02** *
		M4 post 2nd vacc.	**5**	0	53	**0.5**	0	1	0.05
	Native Glycoprotein	Pre vacc.	**2**	0	26	**1.5**	1	3	0.74
		post 1st vacc.	**2**	0	25	**1**	0	4	0.34
		M1 post 2nd vacc.	**4**	0	19	**0.5**	0	2	0.12
		M4 post 2nd vacc.	**2**	0	38	**0**	0	1	0.06
	Whole VZV Antigen	Pre vacc.	**8**	0	206	**9.5**	1	21	0.55
		post 1st vacc.	**16**	0	127	**6**	2	17	0.21
		M1 post 2nd vacc.	**35**	0	140	**7**	1	11	**0.03** *
		M4 post 2nd vacc.	**12**	0	57	**2**	1	3	**0.04** *
Mycophenolate	Glycoprotein E Peptides	Pre vacc.	**2**	0	21	**0**	0	4	**0.03** *
		post 1st vacc.	**3**	0	22	**1**	0	8	0.06
		M1 post 2nd vacc.	**13.5**	0	60	**3**	0	61	0.33
		M4 post 2nd vacc.	**8.5**	0	53	**0**	0	25	**0.045** *
	Native Glycoprotein	Pre vacc.	**2.5**	1	26	**1**	0	4	**0.03** *
		post 1st vacc.	**2.5**	0	25	**1**	0	4	0.21
		M1 post 2nd vacc.	**4**	0	19	**1**	0	14	0.08
		M4 post 2nd vacc.	**3**	0	38	**0**	0	3	**0.03** *
	Whole VZV Antigen	Pre vacc.	**17.5**	1	206	**3**	0	9	**0.002** *
		post 1st vacc.	**19**	0	127	**3**	0	46	**0.01** *
		M1 post 2nd vacc.	**36**	1	140	**18**	0	117	0.24
		M4 post 2nd vacc.	**16.5**	2	57	**1**	0	20	**0.006** *

Median values are highlighted in bold. MIN—minimum; MAX—maximum; M—month; vacc.—vaccination with Shingrix™. Data were compared by using a Mann–Whitney test (* $p < 0.05$).

The remaining clinical parameters had no significant influence on VZV (gE)-specific cellular immunity. However, age tended to correlate negatively with ELISpot responses prior to and post vaccination, i.e., older patients had slightly lower ELISpot responses.

The correlation of the clinical parameters with significant results with the univariate analysis was further examined by using multivariate analysis (Table 5). The VZV (gE)-specific ELISpot results correlated significantly with kidney function (eGFR), with the interval between transplantation and first vaccination, and with sex, diabetes mellitus, and treatment with mycophenolate. For treatment with tacrolimus, only one significant correlation was found, which could also have arisen by chance. Considering long-term immunity (at month 4 after the second vaccination), the interval between transplantation and vaccination had the strongest impact on VZV gE-specific responses ($\chi^2 = 54.0$). Immunity towards the native glycoprotein at month 4 was similarly affected by eGFR, the interval to transplantation, and mycophenolate ($\chi^2 = 39.7$–44.4), and, to a lesser extent, by sex ($\chi^2 = 28.4$) and diabetes mellitus ($\chi^2 = 22.9$). Finally, immunity towards the whole VZV

antigen at month 4 was especially affected by diabetes mellitus (χ^2 = 937.3), followed by sex (χ^2 = 58.9) and interval to transplantation (χ^2 = 29.8).

Table 5. Multivariate analysis of varicella–zoster virus (VZV)-specific ELISpot responses and clinical parameters in 29 kidney transplant recipients.

Antigen	Time Point	eGFR	Interval to KTX [1]	Sex	Diabetes Mellitus	Tacrolimus	Mycophenolate
Glycoprotein E Peptides	Pre vacc.	<0.0001	0.002		<0.0001		<0.0001
	post 1st vacc.						
	M1 post 2nd vacc.						<0.0001
	M4 post 2nd vacc.		<0.0001				
Native Glycoprotein	Pre vacc.	0.02	0.01	<0.0001			0.02
	post 1st vacc.		0.046				
	M1 post 2nd vacc.	<0.0001		<0.0001			
	M4 post 2nd vacc.	<0.0001	<0.0001	0.0001	0.006		<0.0001
Whole VZV Antigen	Pre vacc.	0.01	0.02				0.001
	post 1st vacc.	0.003	<0.0001		<0.0001		
	M1 post 2nd vacc.	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.01
	M4 post 2nd vacc.		0.04	<0.0001	<0.0001		

[1] Interval between kidney transplantation (KTX) and first vaccination (vacc.) with Shingrix™. Data were compared by using multinomial logistic regression, and significant *p* values are indicated. eGFR—estimated glomerular filtration rate.

3.3. Correlation of VZV-Specific Immunity Measured with Various VZV Antigens and at Various Time Points

The Spearman analysis in 29 KTX recipients showed that the ELISpot responses to the different VZV antigens and at the different time points were positively correlated, i.e., immunity to one VZV antigen was predictive of a response to the other two antigens, and data at the different time points were also correlated (Figure 5).

	gE_0	gE_1	gE_2	gE_3	Glyc_0	Glyc_1	Glyc_2	Glyc_3	Whole_0	Whole_1	Whole_2	Whole_3
gE_0		**0.57**	**0.51**	**0.45**	**0.44**	**0.43**	**0.48**	0.35	**0.51**	**0.51**	**0.48**	**0.53**
gE_1	**0.57**		**0.77**	**0.75**	**0.38**	0.31	**0.61**	**0.50**	**0.46**	**0.82**	**0.65**	**0.82**
gE_2	**0.51**	**0.77**		**0.74**	0.34	0.26	**0.69**	**0.54**	0.37	**0.66**	**0.75**	**0.68**
gE_3	**0.45**	**0.75**	**0.74**		0.29	0.22	**0.51**	**0.58**	**0.42**	**0.66**	**0.56**	**0.82**
Glyc_0	**0.44**	**0.38**	0.34	0.29		**0.39**	**0.68**	**0.48**	**0.54**	**0.54**	**0.38**	**0.43**
Glyc_1	**0.43**	0.31	0.26	0.22	**0.39**		**0.44**	0.36	**0.49**	**0.47**	**0.46**	**0.49**
Glyc_2	**0.48**	**0.61**	**0.69**	**0.51**	**0.68**	**0.44**		**0.58**	**0.50**	**0.69**	**0.61**	**0.56**
Glyc_3	0.35	**0.5**	**0.54**	**0.58**	**0.48**	0.36	**0.58**		0.29	**0.56**	**0.53**	**0.66**
Whole_0	**0.51**	**0.46**	0.37	**0.42**	**0.54**	**0.49**	**0.50**	0.29		**0.63**	**0.53**	**0.60**
Whole_1	**0.51**	**0.82**	**0.66**	**0.66**	**0.54**	**0.47**	**0.69**	**0.56**	**0.63**		**0.76**	**0.82**
Whole_2	**0.48**	**0.65**	**0.75**	**0.56**	**0.38**	**0.46**	**0.61**	**0.53**	**0.53**	**0.76**		**0.73**
Whole_3	**0.53**	**0.82**	**0.68**	**0.82**	**0.43**	**0.49**	**0.56**	**0.66**	**0.6**	**0.82**	**0.73**	

$p < 0.05$ $p < 0.01$ $p < 0.001$

Figure 5. Spearman correlation of ELISpot responses towards a peptide pool of glycoprotein E (gE), a native glycoprotein (Glyc), and a whole varicella–zoster virus (Whole) in 29 kidney transplant recipients. Each patient was tested four times, i.e., prior to vaccination (0), after the first vaccination (1), at month 1 after the second vaccination (2), and at month 4 after the second vaccination (3). The numbers indicate the correlation coefficient *r*, which always showed a positive correlation (0.22–0.82). Significant correlations are highlighted in bold; the color indicates the level of significance.

4. Discussion

The current data indicate that vaccination with two shots of Shingrix™ could significantly increase VZV (gE)-specific cellular immunity in KTX recipients, which was detected after in vitro stimulation with a gE peptide pool and a whole VZV antigen. However, as compared to the healthy controls, the cellular responses were lower, as expected. A comparative analysis of various VZV antigens showed that vaccination-induced changes in VZV-specific immunity were most pronounced after stimulation with the gE peptide pool, where we observed an 8.0-fold increase after the second vaccination compared to the baseline. Similar results were observed in a cohort of hematopoietic stem cell transplant recipients, where the gE peptide pool was also most suitable for measuring VZV (gE)-specific vaccination responses [16]. As the zoster vaccine Shingrix™ contains recombinant gE, the most abundant and immune-dominant glycoprotein expressed on the surface of VZV-infected cells [11], this finding appears plausible. It has been shown that gE is a major target for VZV-specific antibody responses [17]. Previously, a strong correlation of glycoprotein-specific antibodies and protection against varicella was shown [18]. In addition, IgG antibodies against gE and IgG antibodies against whole VZV showed positive correlations when analyzing the data qualitatively (positive/negative, 99% agreement) [19] and quantitatively (correlation coefficient of 0.86%) [20]. Similarly to these antibody data, we observed a significant correlation of cellular responses to gE and to whole VZV antigens. Previously, Cassaniti et al. showed that the ELISpot response after stimulation with gE peptides is mainly a CD4 T cell response [21]. This group measured immunity in (unvaccinated) kidney transplant recipients and found an overall range of ELISpot responses that was similar to what we observed in the current study.

There are already data on T cell immunity after vaccination with Shingrix™ in a cohort of 32 kidney transplant recipients [5]. However, immunity was determined through intracellular cytokine staining and detection was performed using flow cytometry after stimulation of CD4 T cells with a pool of peptides covering the gE ectodomain. This study showed a vaccine response rate for cell-mediated immunity of 71% at month 2, defined as an at least twofold increase in responses after two vaccinations. In the current study, we used another method to assess cellular immunity, we tested the samples at month 1 after the second vaccination, and we stimulated PBMCs and not CD4 T cells. Nevertheless, we applied the same criterion, i.e., we determined the percentage of patients with an at least twofold increase in responses after two vaccinations. After stimulation with the gE peptide pool, we found a response rate of 76%. Thus, the data generated by the two different methods fit well.

Moreover, vaccination with Shingrix™ had no effect on allograft function as defined by serum creatinine [5], which could be confirmed by our current data. The correlation of kidney function with immune function is well established [22,23], and therefore, a positive correlation of eGFR with VZV-specific cellular immunity prior to vaccination is in line with current knowledge.

The interval between transplantation and testing and ELISpot results showed a negative correlation, i.e., sooner after transplantation, cellular immune responses were higher. This observation was not expected at first glance. Especially within the first years after transplantation, reactivation of herpesviruses is common [24], and it can be speculated that (subclinical) reactivation caused by immunosuppression leads to an expansion of T cells directed against herpesviruses, such as VZV or cytomegalovirus (CMV). An increased frequency of these specific T cells may result in stronger VZV-specific ELISpot responses at baseline if it is closer to transplantation. This hypothesis is supported by the fact that we observed stronger cellular responses towards CMV in dialysis patients with vs. without immunosuppressive treatment [25] and a higher rate of CMV-specific proliferative responses in hematopoietic stem cell transplant recipients vs. healthy controls [26]. Another unexpected finding, the positive correlation of treatment with tacrolimus or mycophenolate and increased VZV-specific ELISpot responses, may have been caused by a similar phenomenon: (subclinical) VZV reactivation. However, as the majority of patients were treated

with tacrolimus (86%), the observation needs to be interpreted with caution. Of note, two of the patients who did not receive tacrolimus were treated with belatacept and did not develop any cellular responses to vaccination. This finding is in accordance with recent data showing that patients who received belatacept also did not respond to vaccination against SARS-CoV-2 [27–29].

In addition, we could identify male sex as a factor correlated with increased VZV-specific immunity. Consistently with that finding, the previous literature indicated that the incidence of shingles also differed between males and females [6]. The annual rate per 1000 person-years was lower in males (2.6 vs. 3.8, $p < 0.0001$), which could be explained by stronger VZV-specific T cell immunity. Several studies showed sex-dependent immune responses—for example, various concentrations of cytokines or vaccine antibodies [23,30–36]. In females, cytomegalovirus pp65-specific IL-21 ELISpot responses were higher [23] or antibody titers after vaccination against hepatitis B or SARS-CoV-2 virus were increased [30,37]. However, males showed a trend of higher cellular responses towards pneumococcal antigens [38]. It is, therefore, quite possible that VZV-specific immunity is also sex-dependent.

The correlation of diabetes mellitus with impaired cellular responses was expected because hyperglycemia in diabetes is thought to cause dysfunction of the immune response, which fails to control the spread of invading pathogens and makes diabetic subjects more susceptible to infections [39]. We observed a trend of impaired cellular immune response for all VZV antigens and at almost all time points. Since our cohort contained only four patients with diabetes mellitus, this finding did not reach statistical significance for all comparisons.

5. Conclusions

In KTX recipients, vaccination with the adjuvanted, recombinant vaccine Shingrix™, which contains the VZV gE, led to a significant increase in in vitro cellular responses, especially towards VZV gE. This is the first study assessing vaccination efficacy in this setting with ELISpot, an assay that measures active secretion of IFN-γ upon stimulation with VZV antigens. However, as compared to age-matched controls, cellular immune responses after vaccination were weaker in kidney transplant recipients. Furthermore, we could identify sex, kidney function, time point after transplantation, immunosuppressive drugs, and diabetes mellitus as covariates of VZV (gE)-specific cellular vaccination responses; these have not yet been reported.

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