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Relación entre inmunosupresión e inflamación subclínica en biopsias de seguimiento de trasplante renal

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La vida es una sucesión de lecciones que deben ser vividas para ser entendidas.

R.W. Emerson.

ABREVIATURAS

ADEs: Anticuerpos donantes específicos.

ADEs dn: Anticuerpos donante específico de *novo*

AMF: ácido micofenólico.

ARN: Ácido ribonucleico.

BL: *borderline.*

C/D-TAC: concentración/dosis – tacrolimus.

cPRA: porcentaje de anticuerpos reactivos al panel.

dd-cfDNA: ADN libre circulante derivado del donante.

FFPE: fijado con formaldehído y fijado en parafina.

FI/AT: fibrosis intersticial y atrofia tubular.

ICN: Inhibidores de la anticalcineurina.

i-mTOR: inhibidores de la mammalian target de rapamicina.

MMDx: Diagnóstico molecular de rechazo.

MMF: micofenolato de mofetilo.

RAG-Score: Score de rechazo.

RMC: rechazo celular o mediado por células T.

RMA: rechazo mediado por anticuerpos o mediado por células B.

RMAc: rechazo mediado por anticuerpos crónico activo.

RNA-Seq: Secuenciación del transcriptoma entero.

RSC: rechazo subclínico.

RT-MLPA: amplificación de sonda dependiente de ligadura múltiple con transcriptasa inversa.

RT-qPCR: reacción en cadena de la polimerasa con transcriptasa inversa.

TAC: tacrolimus.

TAC —C_0: niveles de tacrolimus en sangre total.

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RESUMEN

La enfermedad renal crónica (ERC) es una de las causas de morbimortalidad más importantes a nivel mundial, con una prevalencia cercana al 10 %. Se estima que para el 2040 se convierta en la quinta causa de muerte a nivel mundial.

Entre las técnicas sustitutivas renales, el trasplante renal (TR) se considera el tratamiento de elección porque ofrece una mejor supervivencia del paciente, una mejor calidad de vida y un menor costo del sistema sanitario en comparación con las técnicas de diálisis. Sin embargo, la supervivencia a largo plazo que ofrece el trasplante no está exento de complicaciones atribuidas a la sobreexposición de la inmunosupresión (neoplasia e infecciones) y a la infraexposición como el rechazo mediado por anticuerpos (RMA), el rechazo mediado por células T (RMC) y la inflamación subclínica que conduce al desarrollo de anticuerpos HLA donante específico de *novo* (*ADEs dn*).

La inmunosupresión de mantenimiento en la mayoría de las unidades de trasplante renal se basa en la combinación de tacrolimus (TAC) y micofenolato mofetilo (MMF), con o sin esteroides en dosis bajas. Una limitación importante de esta estrategia es que el TAC es un fármaco con una ventana terapéutica estrecha, una baja biodisponibilidad (20-30 %), una alta variabilidad intra e interindividual, y además los niveles terapéuticos óptimos (TAC-C_0) durante el seguimiento no han sido definidos adecuadamente. Una baja exposición al TAC durante el primer año se ha asociado a un mayor riesgo de rechazo agudo clínico y subclínico, y a un mayor riesgo de desarrollo de ADEs. Además, estudios previos han mostrado una asociación entre la baja exposición al TAC y una menor supervivencia a largo plazo del injerto. Mientras tanto, una alta exposición al TAC se ha asociado con nefrotoxicidad, infecciones virales y cáncer, entre otras toxicidades. Por este motivo, es importante la monitorización clínica, farmacológica, inmunológica e histológica durante el seguimiento postrasplante.

Durante las últimas décadas la incorporación a la práctica clínica de las biopsias de seguimiento, en diferentes momentos postrasplante, ha permitido caracterizar la presencia de inflamación subclínica y la progresión de la fibrosis, observándose en diferentes estudios su asociación con la exposición a la inmunosupresión, la incompatibilidad HLA y el subsecuente desarrollo de *ADEs dn*.

La monitorización del injerto en el trasplante renal se basa en biomarcadores no invasivos como la creatinina sérica, la proteinuria y los anticuerpos HLA. El desarrollo progresivo de nuevas tecnologías, como el perfil de expresión génica en sangre y

orina, y el ADN libre de células derivado del donante (dd-cfDNA), se han propuesto como nuevas herramientas que pueden contribuir a un mejor entendimiento de los mecanismos inmunopatológicos subyacentes y mejor precisión diagnóstica. De esta manera se podría realizar nuevas intervenciones para prevenir y ofrecer nuevos tratamientos.

El propósito de esta tesis consistió en evaluar la asociación entre la inflamación subclínica del injerto renal (definida como la inflamación microvascular o la inflamación tubulointersticial existente en biopsias de seguimiento); la exposición a la inmunosupresión y la inmunomodulación de la expresión de genes implicados en el rechazo del injerto.

En la presente tesis se describen los resultados de tres publicaciones que abordan los aspectos descritos previamente. En el **primer trabajo,** realizado en receptores de trasplante renal de los cuales se disponía de una biopsia obtenida por seguimiento o por indicación clínica, el objetivo fue determinar la expresión génica diferencial entre biopsias normales, biopsias con rechazo y biopsias con fenotipos incompletos de rechazo. Definimos un score que permitió discriminar las biopsias normales de las biopsias con rechazo (RAG-score). La aplicación de este score en las biopsias con fenotipos incompletos dio resultado positivo en casi todos los casos de rechazo subclínico, en una proporción de pacientes con diagnóstico de fibrosis intersticial y atrofia tubular (FI/AT), y en pacientes con diagnóstico de cambios *borderline* (BL). Es de destacar que el RAG-score se asoció con la supervivencia del injerto de manera independiente al diagnóstico histológico.

En el **segundo trabajo**, evaluamos la asociación entre las lesiones histológicas agudas y crónicas en injertos renales, y la exposición a TAC y su metabolización en pacientes con biopsias de seguimiento secuenciales (a los 3 y 12 meses postrasplante). Observamos que una relación menor de concentración /dosis de tacrolimus (C/D-TAC) se asoció a una mayor progresión de la fibrosis intersticial y atrofia tubular (FI/AT), confirmando que los metabolizadores rápidos presentan más nefrotoxicidad como ha sido descrito previamente. Además, se confirmó que niveles más elevados de TAC se asocian a una menor inflamación en áreas sin fibrosis, pero no en áreas con fibrosis. Por otro lado, observamos que la evaluación del coeficiente de variación de TAC (CV-TAC) y el tiempo en rango terapéutico (TRT) no ofrecen información adicional.

En la misma línea de la investigación, en el **tercer trabajo** evaluamos en pacientes con biopsias de seguimiento, la asociación entre la exposición a TAC y la expresión de transcritos asociados con rechazo descritos por el grupo de Banff (n=308 genes). Mediante la técnica de RT-PCR en tarjetas microfluídicas, observamos que diecinueve de 111 genes asociados al rechazo se correlacionaron con los niveles de tacrolimus valle (TAC-C_0) al momento de la biopsia. Un análisis no supervisado de los datos permitió definir dos grupos de pacientes en función de la expresión génica. Las biopsias con rechazo subclínico y las biopsias con cambios BL coincidieron en el mismo grupo. Además, en este grupo se observó una menor exposición a los niveles de TAC-C_0 y se observó una disminución progresiva de la función renal durante el seguimiento.

ABSTRACT

Chronic kidney disease (CKD) is one of the most significant causes of morbidity and mortality worldwide, with a prevalence close to 10 %. It is estimated that by 2040, it will become the fifth leading cause of death globally. Among renal replacement treatment techniques, kidney transplantation (KT) is considered the treatment of choice because it offers better patient survival, improved quality of life, and lower healthcare system costs compared to dialysis techniques. However, the long-term survival offered by transplantation is not free of complications attributed to overexposure to immunosuppression (neoplasm and infections) and underexposure such as antibody-mediated rejection (ABMR), T-cell-mediated rejection (TCMR), and subclinical inflammation leading to the development of de novo donor-specific HLA antibodies (*dn*DSA).

Maintenance immunosuppression in most kidney transplant units is based on a combination of tacrolimus (TAC) and mycophenolate mofetil (MMF), with or without low-dose steroids. A significant limitation of this strategy is that TAC is a drug with a narrow therapeutic window, low bioavailability (20–30 %), high intra— and interindividual variability, and the optimal therapeutic levels (TAC-C_0) during follow-up have not been determined. Low TAC exposure during the first year has been associated with a higher risk of clinical and subclinical acute rejection and a higher risk of developing *dnDSA.* Additionally, previous studies have shown an association between low TAC exposure and lower long-term graft survival. Meanwhile, high TAC exposure has been associated with nephrotoxicity, viral infections, and cancer, among other toxicities. Therefore, clinical, pharmacological, immunological, and histological monitoring during post-transplant follow-up is important.

In recent decades, the incorporation of follow-up biopsies at different post-transplant times into clinical practice has allowed the characterization of the presence of subclinical inflammation and the progression of fibrosis. Various studies have observed its association with immunosuppression exposure, HLA incompatibility, and the subsequent development of *dnDSA.*

Kidney transplant graft monitoring relies on non-invasive biomarkers such as serum creatinine, proteinuria, and HLA antibodies. The progressive development of recent technologies, such as gene expression profiling in blood and urine and donor-derived cell-free DNA (dd-cfDNA), has been proposed as new tools that can contribute to a better understanding of underlying immunopathological mechanisms and improved

diagnostic accuracy. This could enable new interventions to prevent and offer new treatments.

The purpose of this thesis was to evaluate the association between subclinical inflammation of the kidney graft (defined as microvascular inflammation or tubulointerstitial inflammation present in follow-up biopsies), exposure to immunosuppression, and immunomodulation of the expression of genes involved in graft rejection.

This thesis describes the results of three publications addressing the previously described aspects. The first study, conducted on kidney transplant recipients with available biopsies, obtained through follow-up or clinical indication, aimed to determine the differential gene expression between normal biopsies, rejection biopsies, and biopsies with incomplete rejection phenotypes. We defined a score that allowed us to discriminate between normal biopsies and rejection biopsies (RAG-score). Applying this score to biopsies with incomplete phenotypes yielded positive results in almost all cases of subclinical rejection, a proportion of patients diagnosed with interstitial fibrosis and tubular atrophy (IF/TA), and patients diagnosed with borderline changes (BL). Notably, the RAG-score was associated with graft survival independently of the histological diagnosis.

In the **second article**, we evaluated the association between acute and chronic histological lesions in kidney grafts and TAC exposure and metabolism in patients with sequential follow-up biopsies (at 3— and 12-months post-transplant). We observed that a lower concentration/dose ratio of tacrolimus (C/D-TAC) was associated with greater progression of interstitial fibrosis and tubular atrophy (IF/TA), confirming that fast metabolizers exhibit more nephrotoxicity as previously described. Additionally, it was confirmed that higher TAC levels are associated with less inflammation in non-fibrotic areas, but not in fibrotic areas. On the other hand, we observed that evaluating the TAC coefficient of variation (CV-TAC) and time in the therapeutic range (TTR) does not provide additional information.

In line with the investigation, in the **third article**, we evaluated the association between TAC exposure and the expression of rejection-associated transcripts described by the Banff group (n = 308 genes) in patients with follow-up biopsies. Using RT-PCR on microfluidic cards, we observed that nineteen of 111 rejection-associated genes correlated with trough tacrolimus levels (TAC-C_0) at the time of biopsy. An unsupervised analysis of the data allowed the definition of two patient groups based on

gene expression. Subclinical rejection biopsies and biopsies with BL changes coincided in the same group. Additionally, this group showed lower exposure to TAC-C_0 levels and a progressive decline in renal function during follow-up.

1. INTRODUCCIÓN

1.1 TRASPLANTE RENAL.

1.1.1 Generalidades e historia del Trasplante renal.

A inicios del siglo XX, la enfermedad renal crónica (ERC), fue una causa importante de mortalidad [1]. La incidencia de ERC es variable de acuerdo con las zonas geográficas, a pesar de que la magnitud y el impacto de la enfermedad renal están mejor definidos en los países desarrollados, la evidencia sugiere que en los países en desarrollo existe una carga de la enfermedad similar o incluso mayor que en los primeros.

La ERC se ha vinculado con un incremento del riesgo cardiovascular, lo que repercute negativamente en la calidad de vida y el pronóstico de los pacientes. Las dos principales terapias de reemplazo renal son la diálisis y el trasplante renal. Aunque la diálisis ha avanzado en las últimas décadas, el trasplante renal proporciona al paciente una mejor supervivencia de forma global [2,3]. El desafío principal reside en las extensas listas de espera y la disponibilidad limitada de órganos.

Los inicios del siglo XX marcan el comienzo de la historia del trasplante renal, Ullman en 1902 llevó a cabo un autotrasplante de un riñón de perro desde su posición anatómica normal a los vasos del cuello. En el mismo año y también en Viena, Von de Castello realizó un trasplante renal entre perros. Sin embargo, se considera que el primer trasplante renal en humano se realizó en 1906, por el médico francés Mathieu Jaboulay. Se trataba de una mujer que presentaba un síndrome nefrótico, el riñón provenía de un cerdo y la anastomosis realizada fue sobre la arteria humeral y la vena cefálica, funcionó durante una hora.

En 1912, Alexis Carrel, discípulo de Jaboulay, demostró la importancia de la sutura vascular para la viabilidad de los trasplantes de órganos sólidos, este avance le valió el Premio Nobel de Medicina en dicho año. En 1933, el ruso Yu Voronoy llevó a cabo el primer trasplante de donante cadáver, pero fracasó pocos días después debido a una incompatibilidad ABO entre el donante y el receptor [4]. Todo esto dejó en claro que existía un componente inmunológico en el trasplante y que si se quería tener éxito se debería profundizar en el conocimiento de la inmunología y su repercusión en el trasplante. Los primeros trasplantes renales exitosos se realizaron en la década de los 50, en París y Boston. En 1953, el profesor Hamburger del grupo de París llevó a cabo el primer trasplante de donante vivo genéticamente relacionado. En 1954, el grupo de Boston, liderado por el doctor Murray realizó el primer trasplante renal entre

gemelos univitelinos [5]. Cinco años más tarde se iniciaron los trasplantes entre hermanos no idénticos. En 1963 se realizó el primer trasplante de donante en muerte encefálica, lo que sentó un precedente para creación normas de carácter legal que permitieran la extracción y trasplante de órganos.

En España, en 1979, se promulgó la ley de 27 de octubre 30/179, donde se tomaba en consideración este punto. En la década del 60, se realizó en España el primer trasplante renal en el Hospital Clinic de Barcelona, en el que participaron los doctores José María Gil-Vernet, Antoni Caralps y Jordi Vives [6].

Teniendo en cuenta las mejoras en cuanto a la técnica, el problema se focaliza en el mantenimiento de la función renal. A partir de los años 60, el principal objetivo fue encontrar la estrategia para disminuir la respuesta inmunológica del receptor, para ello, se empleó, por ejemplo, la irradiación linfática total, la que presentaba una alta mortalidad y fue desestimada.

Previamente, en esa línea inmunológica, se había descrito el complejo mayor de histocompatibilidad (CMH), en ratones, por Peter Gorer, lo cual permitió posteriormente asociar que el rechazo en ratones estaría dado por incompatibilidades en algunos antígenos. En años siguientes se pudo definir el cromosoma donde se codifican los antígenos leucocitarios humanos (HLA) A, B y C, y más adelante P. Terasaki desarrolló la prueba de citotoxicidad dependiente de complemento, la que demostró la importancia de la prueba cruzada entre donante y receptor para evitar el rechazo hiperagudo [7,8].

Por otra parte, se empezaron a utilizar fármacos con finalidad de inmunosuprimir y disminuir las probabilidades de rechazo. A inicios de los 60, se introdujo la azatioprina como tratamiento inmunosupresor oral; y posteriormente, se usó en combinación con corticoides para tratamiento de episodios de rechazo agudo. En la década de los 70 se añadió la ciclosporina y en los 90 se añadieron el micofenolato mofetil (MMF) y el tacrolimus (TAC); todos estos avances a nivel farmacológico contribuyeron a mejorar los resultados en supervivencia de los trasplantes.

1.1.2 Situación actual de la enfermedad renal crónica y el trasplante renal en nuestro medio.

Cada año se realiza un informe por parte de la ERA-EDTA en el que se detallan los datos referentes a la situación a nivel europeo de la ERC. El informe del 2021 reportó que la incidencia de inicio de tratamiento renal sustitutiva fue de 145, por millón de población (pmp) (figura 1). La modalidad de elección de tratamiento renal fue la hemodiálisis 83 %, seguida de la diálisis peritoneal 11 % y el trasplante vivo anticipado con un 5 %. La prevalencia fue de 1040 pmp, con un 56 % de los pacientes en hemodiálisis (HD), un 5 % en diálisis peritoneal (DP) y 39 % trasplantados. La tasa de trasplante en 2021 fue de 37 pmp, mejoría notable tras la pandemia del SARS-CoV-22 [9].

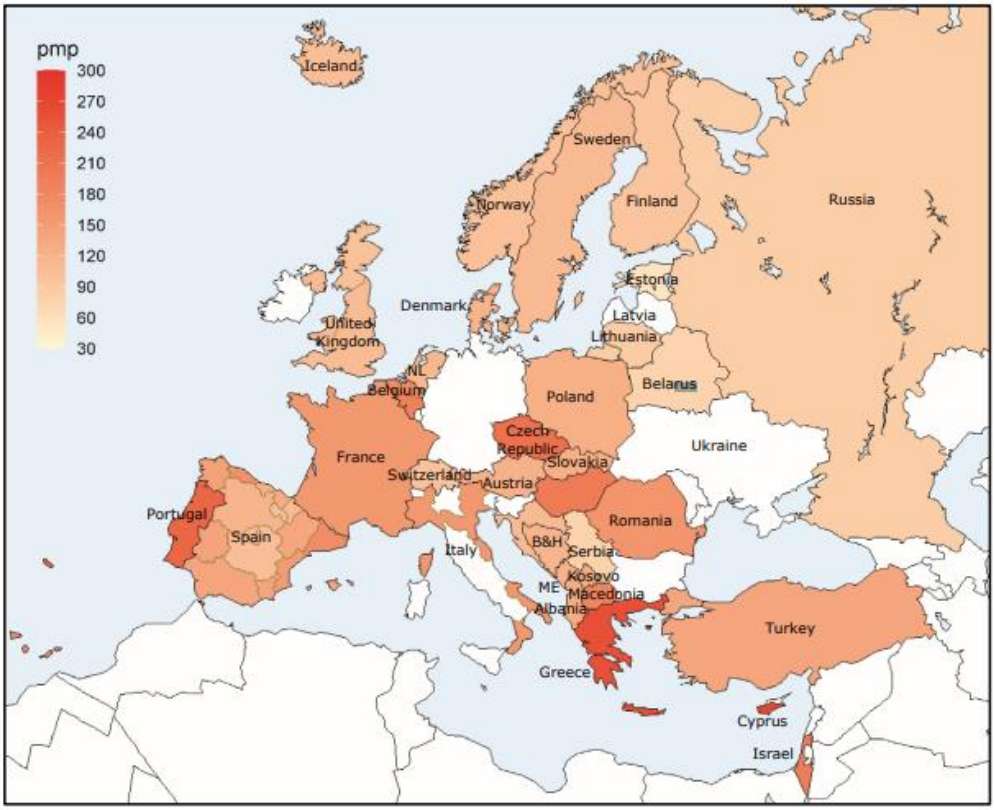

Figura 1. Incidencia de ERC (pmp) en 2021 en UE [9]

Si se evalúan los resultados a nivel de España, de acuerdo con el Registro Español de Enfermos Renales del 2022, la incidencia de pacientes renales se encuentra por encima de 150 pmp, manteniéndose en valores cercanos previo a la pandemia (figura 2) [10].

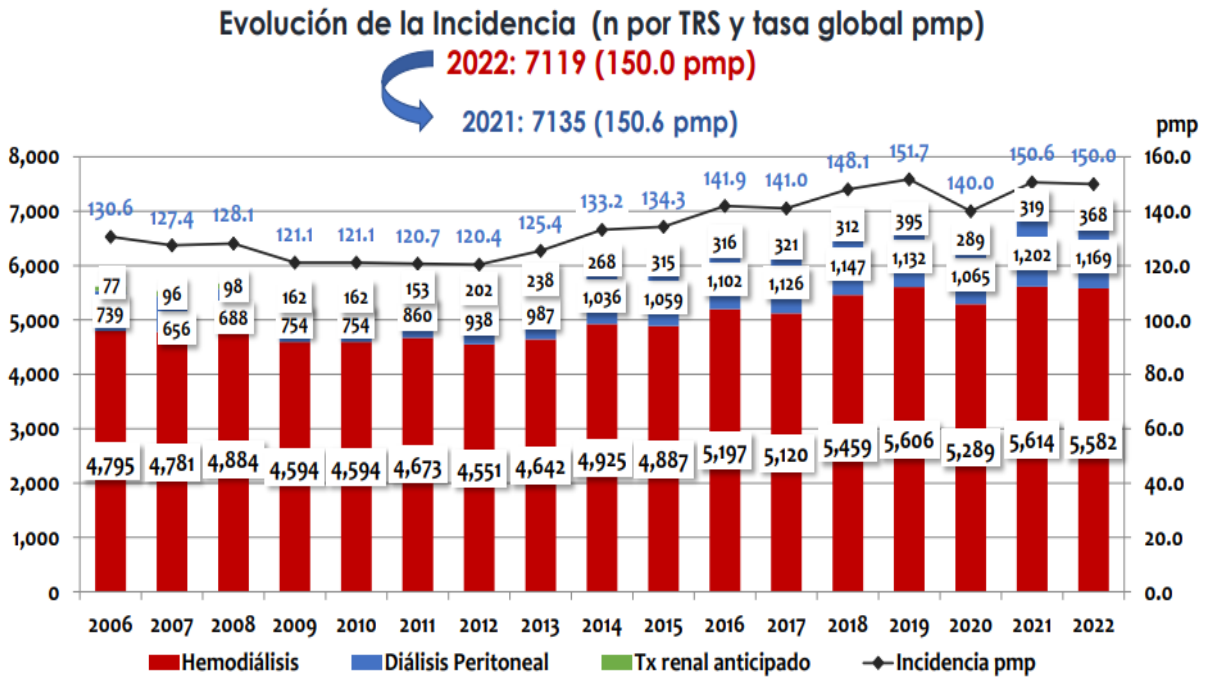

Figura 2. Incidencia de TSR y tasa global pmp en España [10].

La prevalencia de la ERC ha aumentado por encima de los 1400 pmp. En cuanto a la prevalencia de los trasplantes se sitúan en torno al 55%, mientras que en el resto de las modalidades se mantiene estable (figura 3).

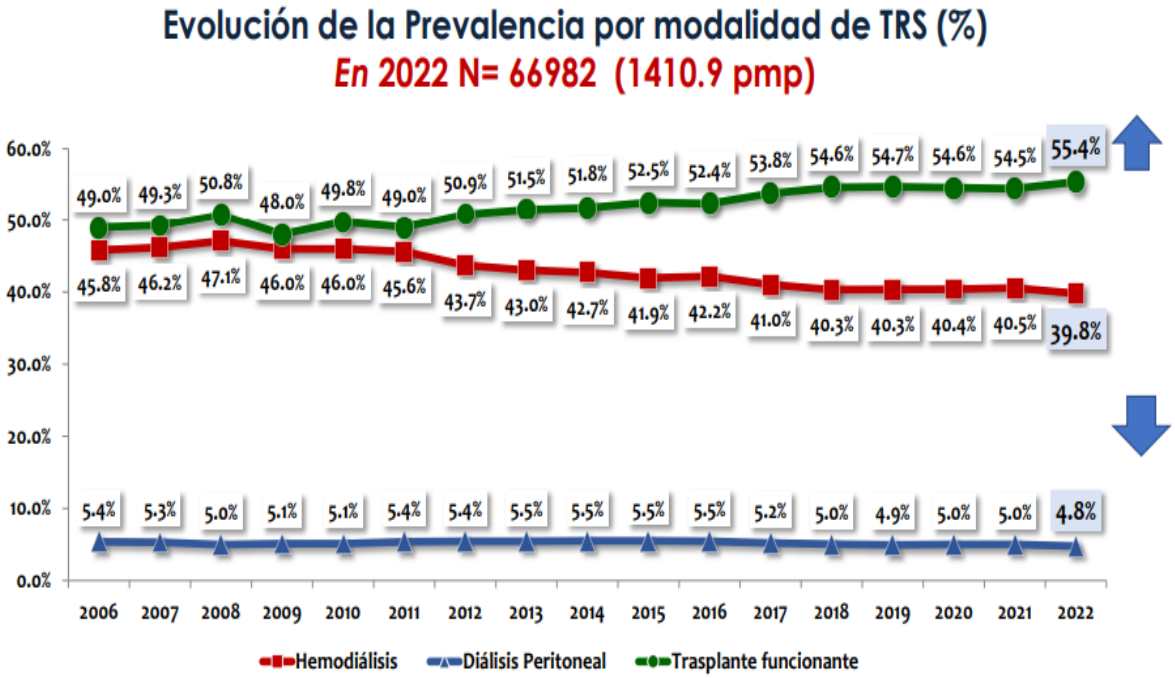

Figura 3. Prevalencia de TSR en España de acuerdo con modalidad realizada (%)[10]

En lo que respecta al trasplante renal en España, en el 2023 (figura 4), se realizaron 3.688 trasplantes renales (8 % más que el año 2022) alcanzando un récord histórico (figura 4) [11]. Es de mencionar que en torno al 25 % de estos trasplantes fueron realizados en Cataluña, lo que demuestra que es una región pionera en trasplante renal.

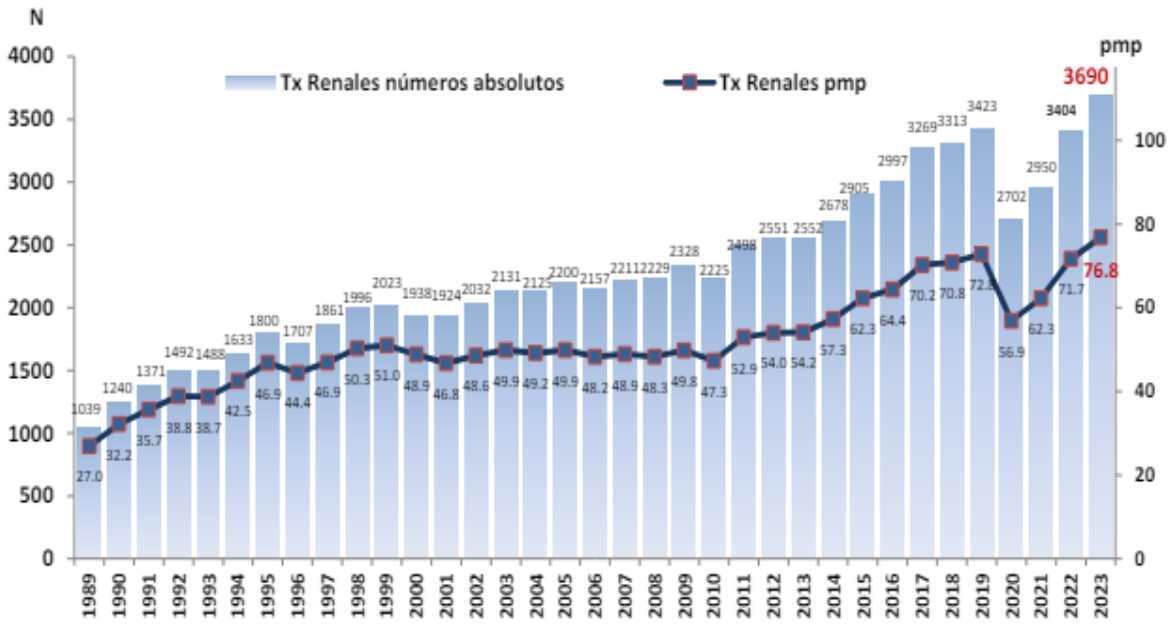

Figura 4. Evolución de la actividad de trasplante donante en España (número absoluto y pmp) durante el período 1989-2023 [11].

1.2 MONITORIZACIÓN POSTRASPLANTE.

La monitorización posterior al trasplante se basa en métodos no invasivos como la determinación de la creatinina, proteinuria, filtrado glomerular, de niveles de inmunosupresores, y anticuerpos anti-HLA, así como métodos invasivos como lo es la biopsia renal.

1.2.1 Monitorización histológica

La biopsia del injerto renal se considera el "Gold standard" para el diagnóstico y monitorización del injerto renal [12]. Las biopsias se efectúan con la administración de anestésico local y se emplean agujas de calibre 14, 16 o 18 G [13]. En la actualidad, al realizarse de manera eco-guiada, se considera un procedimiento seguro, pudiendo realizarse de forma ambulatoria [14,15]. No existe un claro consenso sobre los criterios de contraindicación para la realización de la biopsia del injerto ni el tiempo de observación tras el procedimiento. En algunas series se considera un período de observación entre 4 a 8 horas, durante las cuales se realiza la monitorización del paciente [14,16]. El índice de complicaciones graves se ha descrito inferior al 1 %, considerándose un procedimiento seguro [16,17].

1.2.1.1 Criterios de Banff.

Hace tres décadas, un equipo de médicos dedicados al trasplante y patólogos, liderados por Solez, Keow y Racusen llevaron a cabo un primer consenso sobre los criterios histológicos para la clasificación de la patología del injerto renal. El grupo de Banff, se reúne cada dos años para deliberar sobre nuevas modificaciones en las clasificaciones de diversas patologías que afectan al injerto renal, publicando un documento actualizado tras cada sesión [18]. Desde 1991 en adelante, los diagnósticos histopatológicos relacionados al injerto se rigen de acuerdo con las directrices determinadas por este grupo. En 1993 el documento propuso la evaluación mediante una escala ordinal de las siguientes lesiones elementales: inflamación intersticial (i), tubulitis (t), endarteritis (v), y glomerulitis (g), glomerulopatía del trasplante (cg), hialonosis arteriolar (ah), engrosamiento fibroso vascular (cv), fibrosis intersticial (ci), y atrofia tubular (ct) [18]. En la reunión de 1997 se redactó un documento en donde se especifican las características de las muestras renales para ser consideradas óptimas; y se concluyó que la biopsia debía tener un mínimo de 10 glomérulos y 2 arterias para considerarse adecuada [19].

En la reunión de 2001 se definieron los criterios de rechazo mediado por anticuerpos (RMA), debiéndose cumplir criterios histológicos (inflamación microvascular [MVI]), criterios inmunohistológicos (tinción positiva para C4d en capilares peritubulares) y la presencia de ADEs para su diagnóstico. Además, se presentaron por primera vez resultados de estudios moleculares aplicados a biopsias de trasplante [20].

En el año 2003, se definió el rechazo mediado por anticuerpos crónico activo (RMAc) [21]. En el 2007 se añadió la categoría de inflamación total (ti) en áreas con y sin cicatriz, relacionado con la evolución de infiltrados intersticiales. En el 2013 se redefinieron los criterios de RMA, aceptándose el RMA C4d negativo [22]. Se dio nuevo énfasis a la importancia de la microscopía electrónica, con la introducción de criterios específicos para el RMAc y se añadió oficialmente por primera vez el diagnóstico molecular a la clasificación de Banff [23]. En 2015 se reconoce la importancia de anticuerpos no HLA en el rechazo y se propone identificar genes que pudiesen tener relación con los principales fenotipos clínicos de RMC y RMA [24].

En 2017 se definió y se determinaron los criterios de inflamación en áreas de fibrosis intersticial/atrofia tubular (i-FI/AT), lo cual permitió definir los criterios de RMC crónico. Además, se revisó un primer borrador de un listado de genes para el diagnóstico molecular de rechazo, se presentó además nueva tecnología Nanostring que permitía cuantificar transcripciones múltiples a partir de biopsias fijadas con formalina e incluidas en parafina (FFPE) lo que permitía aprovechar la misma muestra de tejido renal ya analizada [25].

En 2019 se redefinieron los criterios de cambios *borderline* (BL), considerándose presencia de inflamación intersticial que involucra del 10 % al 25 % de la corteza (i1) con al menos tubulitis leve (t> 0) (tabla 1) [26].

Tabla 1. Clasificación de los diagnósticos histológicos de Banff 2019 [26]

TABLE 4 Updates of 2019 Banff classification for ABMR, borderline changes, TCMR, and polyomavirus nephropathy. All updates in boldface type[a]

Category 1: Normal biopsy or nonspecific changes
Category 2: Antibody-mediated changes
Active ABMR; all 3 criteria must be met for diagnosis
1. Histologic evidence of acute tissue injury, including 1 or more of the following: • Microvascular inflammation (g > 0 and/or ptc > 0), in the absence of recurrent or de novo glomerulonephritis, although in the presence of acute TCMR, borderline infiltrate, or infection, ptc ≥ 1 alone is not sufficient and g must be ≥ 1 • Intimal or transmural arteritis (v > 0)[b] • Acute thrombotic microangiopathy, in the absence of any other cause • Acute tubular injury, in the absence of any other apparent cause
2. Evidence of current/recent antibody interaction with vascular endothelium, including 1 or more of the following: • Linear C4d staining in peritubular capillaries or medullary vasa recta (C4d2 or C4d3 by IF on frozen sections, or C4d > 0 by IHC on paraffin sections) • At least moderate microvascular inflammation ([g + ptc] ≥2) in the absence of recurrent or de novo glomerulonephritis, although in the presence of acute TCMR, borderline infiltrate, or infection, ptc ≥ 2 alone is not sufficient and g must be ≥1 • Increased expression of gene transcripts/classifiers in the biopsy tissue strongly associated with ABMR, if thoroughly validated
3. Serologic evidence of circulating donor-specific antibodies (DSA to HLA or other antigens). C4d staining or expression of validated transcripts/classifiers as noted above in criterion 2 may substitute for DSA; however thorough DSA testing, including testing for non-HLA antibodies if HLA antibody testing is negative, is strongly advised whenever criteria 1 and 2 are met
Chronic active ABMR; all 3 criteria must be met for diagnosis
1. Morphologic evidence of chronic tissue injury, including 1 or more of the following: Transplant glomerulopathy (cg > 0) if no evidence of chronic TMA or chronic recurrent/de novo glomerulonephritis; includes changes evident by electron microscopy (EM) alone (cg1a) Severe peritubular capillary basement membrane multilayering (ptcml1; requires EM) Arterial intimal fibrosis of new onset, excluding other causes; leukocytes within the sclerotic intima favor chronic ABMR if there is no prior history of TCMR, but are not required
2. Identical to criterion 2 for active ABMR, above
3. Identical to criterion 3 for active ABMR, above, including strong recommendation for DSA testing whenever criteria 1 and 2 are met. **Biopsies meeting criterion 1 but not criterion 2 with current or prior evidence of DSA (posttransplant) may be stated as showing chronic ABMR, however remote DSA should not be considered for diagnosis of chronic active or active ABMR**
Chronic (inactive) ABMR
1. cg > 0 and/or severe ptcml (ptcml1)
2. Absence of criterion 2 of current/recent antibody interaction with the endothelium
3. Prior documented diagnosis of active or chronic active ABMR and/or documented prior evidence of DSA

TABLE 4 (Continued)

C4d staining without evidence of rejection; all 4 features must be present for diagnosis[c]
1. Linear C4d staining in peritubular capillaries (C4d2 or C4d3 by IF on frozen sections, or C4d > 0 by IHC on paraffin sections)
2. Criterion 1 for active or chronic active ABMR not met
3. No molecular evidence for ABMR as in criterion 2 for active and chronic active ABMR
4. No acute or chronic active TCMR, or borderline changes
Category 3: Borderline (Suspicious) for acute TCMR
Foci of tubulitis (t1, t2, or t3) with **mild interstitial inflammation (i1)**, or mild (t1) tubulitis with moderate-severe interstitial inflammation (i2 or i3)
No intimal or transmural arteritis (v = 0)
Category 4: TCMR
Acute TCMR
Grade IA: Interstitial inflammation involving >25% of non-sclerotic cortical parenchyma (i2 or i3) with moderate tubulitis (t2) involving 1 or more tubules, not including tubules that are severely atrophic[d]
Grade IB: Interstitial inflammation involving >25% of non-sclerotic cortical parenchyma (i2 or i3) with severe tubulitis (t3) involving 1 or more tubules, not including tubules that are severely atrophic[d]
Grade IIA: Mild to moderate intimal arteritis (v1), with or without interstitial inflammation and/or tubulitis
Grade IIB: Severe intimal arteritis (v2), with or without interstitial inflammation and/or tubulitis
Grade III: Transmural arteritis and/or arterial fibrinoid necrosis involving medial smooth muscle with accompanying mononuclear cell intimal arteritis (v3), with or without interstitial inflammation and/or tubulitis
Chronic active TCMR[e]
Grade IA: Interstitial inflammation involving >25% of sclerotic cortical parenchyma (i-IFTA2 or i-IFTA3) AND > 25% of total cortical parenchyma (ti2 or ti3) with moderate tubulitis (t2 or **t-IFTA2**) involving 1 or more tubules, not including severely atrophic tubules[d]; other known causes of i-IFTA should be ruled out
Grade IB: Interstitial inflammation involving >25% of sclerotic cortical parenchyma (i-IFTA2 or i-IFTA3) AND > 25% of total cortical parenchyma (ti2 or ti3) with severe tubulitis (t3 or **t-IFTA3**) involving 1 or more tubules, not including severely atrophic tubules[d]; other known causes of i-IFTA should be ruled out
Grade II: Chronic allograft arteriopathy (arterial intimal fibrosis with mononuclear cell inflammation in fibrosis and formation of neointima). This may also be a manifestation of chronic active or chronic ABMR or mixed ABMR/TCMR
Category 5: polyomavirus nephropathy[f]
PVN Class 1 **pvl 1 and ci 0-1**
PVN Class 2 **pvl 1 and ci 2-3 OR** **pvl 2 and ci 0-3 OR** **pvl 3 and ci 0-1**
PVN Class 3 **pvl 3 and ci 2-3**

Durante este consenso se sentaron las bases para el desarrollo del proyecto: the Banff-Human Organ Transplant (B-HOT); que consistió en el desarrollo de un panel de genes para el diagnóstico molecular de rechazo utilizando tecnología de Microarrays y NanoString (figura 5) [27,28].

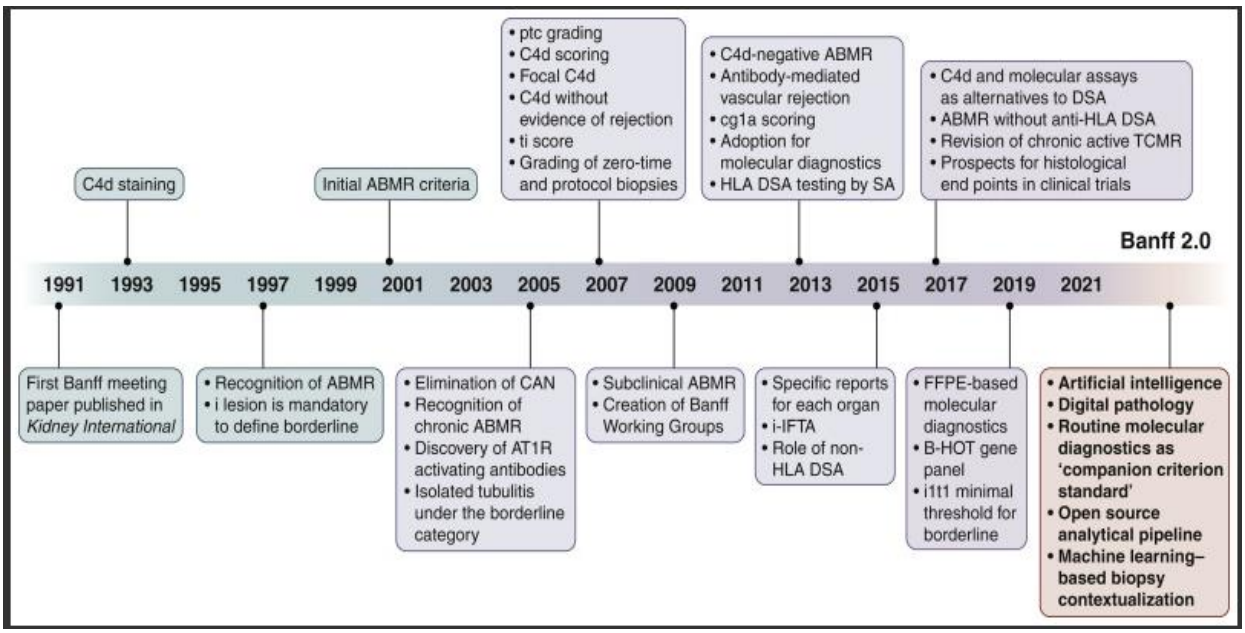

Figura 5. Evolución de la clasificación de Banff 1991 a 2021: conceptos y cambios importantes [28]

En 2022, la evaluación del rechazo se centró en la inflamación microvascular. Se definieron términos como “probable RMA” para casos con ADEs, pero sin inflamación microvascular, y se identificó el fenotipo de “inflamación microvascular con C4d negativo y sin ADEs”. Además, se establecieron distintos grupos de trabajo en trasplante dedicados al estudio del diagnóstico molecular del rechazo, asociándolo con los hallazgos histológicos para mejorar la comprensión de los mecanismos subyacentes de rechazo y una mejor precisión diagnóstica [29].

El siguiente reto está siendo correlacionar la histología, con el diagnóstico molecular y la implementación de algoritmos diagnósticos utilizando la inteligencia artificial (IA).

1.2.1.2 Biopsias de injerto renal de seguimiento.

En los años 90 en diversas unidades de trasplante renal tanto europeas como norteamericanas, se consideró realizar biopsias de injerto renal de pacientes estables, las cuales fueron denominadas biopsias de protocolo o de seguimiento [18].

Rush et al.[30], fueron pioneros en efectuar estudios en pacientes con trasplantes renales que presentaban función renal estable, identificando lesiones histopatológicas como infiltrado inflamatorio intersticial y tubulitis. Este hallazgo fue denominado rechazo subclínico (RSC). Desde entonces, los esfuerzos se han centrado en comprender cómo estas lesiones se relacionan con el pronóstico del trasplante. En la actualidad, las biopsias de seguimiento de forma rutinaria en algunos centros, pero no de forma generalizada. Habitualmente se realizan: una biopsia precoz, entre los tres y seis meses postrasplante,y una biopsia tardía entre el primer y segundo año postrasplante [31]. Su realización de forma ambulatoria es segura [32].

1.2.1.2.1 Rechazo subclínico (RSC) en biopsias de seguimiento.

Existen dos tipos de RSC en biopsias de seguimiento: el celular o mediados por células T (destaca la inflamación intersticial y la tubulitis) y el humoral o mediados por anticuerpos (inflamación microvascular (MVI): glomerulitis y capilaritis con presencia o no de depósitos C4d en capilares peritubulares).

1.2.1.2.1.1 Rechazo celular e Inflamación tubulointersticial.

El 75 % de las biopsias de seguimiento con hallazgos compatibles con inflamación tubulointersticial corresponden a cambios BL; mientras que el tercio restante corresponde a un diagnóstico de RMC. Se ha observado que las lesiones vasculares aisladas suelen ser excepcionales [33] y habitualmente estas lesiones tubulointersticiales se asocian poco a la presencia de anticuerpos donante específico.

Nankivell et al. analizaron a 119 receptores de trasplante riñón-páncreas mediante biopsias sucesivas, observaron cerca de un 61 % de RMC al mes del trasplante y un 45 % a los tres meses, lo que sugiere que la posibilidad de RMC es menor conforme transcurre el trasplante [34]. Las biopsias realizadas a los siete días y a las cuatro semanas postrasplante mostraron una menor tasa de rechazo en aquellos pacientes con tratamiento individualizado, de acuerdo con su perfil inmunológico (32.6 % *versus* un 57.2 %) [35]. En el estudio TRANSFORM se evaluó la no inferioridad de una pauta de i-mTOR junto con una dosis reducida de ICN *versus* ácido micofenólico (MPA) y dosis

estándar de ICN (EVE n=1022 *versus* MPA n= 1015). En este estudio se confirmó la no inferioridad del EVE frente al MPA [36.37]. Es de importante destacar que, con las combinaciones actuales de inmunosupresión, basadas en TAC y MMF o i-mTOR la tasa de RSC se sitúa entre el 10-15 % durante el primer año del trasplante.

La inmunosupresión juega un papel importante en el proceso; existen diversos estudios donde se ha observado una mayor incidencia de RSC tubulointersticial en los grupos de pacientes con retirada temprana de corticoides [38,39]. La reducción de episodios de RSC ha sido viable principalmente gracias a la optimización de la inmunosupresión [40,41]. Flechner et al.[42], realizaron un estudio donde se evaluó la terapia libre de inhibidores de la calcineurina (ICN) versus el régimen basado en inhibidores del mTOR (i-mTOR), concluyendo que el grupo libre de ICN presentaba una mayor incidencia de rechazo. Moreso et al. [43], analizaron 435 biopsias de seguimiento realizadas durante el primer trimestre postrasplante. En este trabajo se observó una tasa de rechazo celular subclínico del 56 % en los pacientes con terapia libre de ICN *versus* un 16 % en aquellos que estaban en tratamiento con TAC. El París Transplant Group, liderado por Loupy, realizó un estudio en donde se incluyeron 1001 pacientes a los que se le realizó una biopsia al año de trasplante y se observó que aquellos que presentaron RSC provenían en su mayoría de donantes fallecidos (88 % *versus* un 71 %, p=0.02) [44].

Choi et al. [45], llevaron a cabo un estudio en donde se analizaron 304 biopsias realizadas a los 14 días después del trasplante de donante vivo, de estas, 195 provinieron de donantes vivos genéticamente relacionados y 109 de donantes vivos genéticamente no relacionados. Se observó una mayor incidencia de RSC en los receptores de donantes genéticamente no relacionados en comparación con los relacionados genéticamente (19 % *versus* 10 %, $p < 0.05$). Además de las características del donante, también se deben considerar las del receptor, las cuales están fundamentalmente relacionadas con la inmunología. El desarrollo de anticuerpos donantes específicos se ha asociado a un mayor número de incompatibilidades HLA y/o presencia de un cPRA elevado; lo cual conlleva finalmente a inflamación tanto clínica como subclínica en biopsias de seguimiento.

En el mismo trabajo de Choi, se observa que aquellos pacientes que presentan menor riesgo de rechazo fueron aquellos que no presentaban incompatibilidades HLA DR (0 HLA DR mm 2,7 %, *versus* 1 HLA DR mm 15,4 % *versus* 2 HLA DR mm 20,8 %). En el trabajo realizado por García-Carro et al.[46], se observó que un mayor número de incompatibilidades HLA DR (OR 1.95; IC 95 % 1.09-3.49), y la inflamación subclínica

con o sin lesiones crónicas tubulointersticiales es un factor independiente del desarrollo de *ADEs dn.*

Mehta et al. [47], compararon el impacto de la tubulitis subclínica con o sin inflamación intersticial, con la inflamación intersticial aislada en biopsias normales a los 3 meses postrasplante, concluyendo que la tubulitis aislada o relacionada con inflamación presenta una mayor incidencia de RMC (subclínico y clínico) al año de trasplante en comparación a biopsias normales (31 % *versus* el 17 %), así como al desarrollo de *ADEs dn* (6 % *versus el* 0.7 %). La presencia de rechazo subclínico, especialmente cuando se asocia con factores inmunológicos desfavorables, puede llevar a un peor pronóstico del injerto a largo plazo.

1.2.1.2.1.2 Rechazo mediado por anticuerpos.

Por muchos años el rechazo celular fue uno de los mayores problemas en lo que respecta al trasplante, sin embargo, en la actualidad lo es el rechazo mediado por anticuerpos, sobre todo el rechazo mediado por anticuerpos crónico activo, siendo la principal causa de pérdida del injerto [48].

En el trabajo de Mengel et al. [49], se describe cómo el desarrollo de ADEs genera el daño a nivel de la microcirculación del injerto renal. Se cree que no todos los ADEs son capaces de producir rechazo; esto depende, entre otros motivos, de su capacidad de fijar complemento. Los anticuerpos capaces de fijar complemento, como son las subclases IgG1 e IgG3 son deletéreos para el injerto renal [50]. En un trabajo liderado por Loupy et al. [51], se observó que los casos en los que existía mayor riesgo de pérdida del injerto fueron en aquellos que, tras el tratamiento del RMA fijaban complemento C1q.

El espectro del RMA abarca desde el rechazo hiperagudo, actualmente improbable debido a la contraindicación del trasplante con prueba cruzada positiva, hasta el RMAc incluyendo fenotipos incompletos como la presencia de C4d sin signos evidentes de RMA. La lesión característica es la presencia de inflamación microvascular. En Banff del 2013 se redefinió RMA activo o agudo en donde podrían no existir depósitos de C4d, pero debería existir interacción de ADEs a nivel de la microcirculación o la expresión de genes de rechazo.

La importancia o equivalencia de los depósitos de C4d, es bastante variable. Loupy et al. [52], analizaron las biopsias del seguimiento a los tres meses de una cohorte de receptores sensibilizados previamente, y demostraron una prevalencia del 49 % de

RMA-C4d negativo, frente al 31 % de RMA-C4d positivo. El grupo RMA-C4d negativo desarrolló posteriormente más fibrosis, glomerulopatía y peor función al año del trasplante. Orandi et al. [53], en cambio, no observaron diferencias en cuanto a supervivencia entre grupo con C4d positivo y negativo. A pesar de los diferentes resultados, se considera que la presencia de C4d positivo se corresponde a la activación del complemento y a una peor evolución del injerto renal en biopsias de causa [54]. En pacientes con ADE al momento de la biopsia de seguimiento, se observó, hasta en un 50 % de los casos, la presencia de inflamación microvascular con C4d positivo; en estos pacientes, las biopsias al año mostraron persistencia de la inflamación junto con progresión de lesiones crónica [12,52,55].

Moreso et al. [56], analizaron 517 receptores con biopsias de seguimiento, se observó que 109 de estos se someten a una segunda biopsia por disfunción del injerto y de estos 44 presentaban RMA. El grupo de RMA tenía una mayor incidencia de RSC en la primera biopsia que los que presentaban cambios de FI/AT sin signos de rechazo (52.3% *versus* el 28 %, p= 0.0253).

1.2.1.2.2 Lesión crónica no inmunológica.

A diferencia de las lesiones inflamatorias, la progresión de las lesiones de fibrosis intersticial y atrofia tubular se consideran lesiones crónicas, son irreversibles y se relacionan directamente con la pérdida del injerto renal, ya que se suman a las lesiones de glomeruloesclerosis y a las lesiones vasculares. En diferentes estudios se ha demostrado que la presencia de FI/AT se observa hasta en cerca del 50 % de los trasplantes al año [57]. Isoniemi et al. [58], realizaron una de las primeras publicaciones de biopsia de seguimiento a los dos años de trasplante, en donde se observa que cerca del 50 % de los pacientes presentaban lesiones crónicas y esto condiciona una peor evolución del injerto.

En 2003, Nankivell et al. [59], evaluaron la prevalencia de fibrosis intersticial moderada o grave, que fue a los 5 años del 66 %, mientras que en el trabajo de Stegall et al. [60], fue del 17 %. Se considera que estas lesiones pueden provenir del propio órgano, visibles en las biopsias preimplante; así como debido a la isquemia, reperfusión y la necrosis tubular aguda que sufre el injerto [59,61].

Existen varios trabajos en donde en los regímenes libres de ICN, ya sea basado en i-mTOR o Belatacept, se observa una menor progresión de FI/AT [62–64]. En los estudios BENEFIT y BENEFIT EXT se observó que los pacientes tratados con Belatacept

mostraron una menor progresión de FI/AT en comparación con los pacientes tratados con CsA (18 % *versus* el 32 %) [65,66]. En un estudio prospectivo donde los pacientes fueron aleatorizados a cuatro esquemas inmunosupresores diferentes, se encontró que los regímenes que combinaban un ICN con un inhibidor de mTOR, presentaban menos fibrosis, evaluada mediante una biopsia de vigilancia a los cinco años [67]. Lo mismo que se observa en el trabajo de Naesens et al., donde niveles altos de TAC (niveles > 13 ng/ml entre los 0-3 meses y niveles >10.5 ng/ml 3-12 meses), no se asociaron con lesiones consideradas como nefrotoxicidad por ICN [68]. Torres et al. [69], en su trabajo, argumentan que durante los primeros meses después del trasplante, es esencial mantener una dosis adecuada de medicamentos inmunosupresores y que no es recomendable reducirlos prematuramente.

1.2.1.2.3 Cambios borderline.

La categoría de cambios *borderline* definida por el consenso de Banff tiene un significado incierto. Si bien puede representar un verdadero RMC, también puede ser el correlato morfológico de una lesión renal aguda asociada con isquemia-reperfusión u otros tipos de lesión. Los criterios para su diagnóstico han ido cambiando con el tiempo; en la primera reunión de Banff, esta categoría incluía biopsias con tubulitis leve (t1) asociada con inflamación intersticial de leve a grave (i1-i3) ***[18]***. En las reuniones de 2005-2007, se actualizó la clasificación y también se incluyeron en esta categoría las biopsias de trasplante renal que mostraban tubulitis de leve a grave (t1-t3) sin inflamación intersticial (i0) ***[70]***.

Recientemente, la evidencia sugiere que los pacientes con una puntuación de biopsia ≥ i1t1 tienen un peor pronóstico que los pacientes con i0t1, lo que pone en duda los umbrales de diagnóstico actuales [71]. Además, los enfoques terapéuticos hacia los cambios *borderline* varían en diferentes entornos clínicos. El diagnóstico de cambios *borderline* en las biopsias por indicación conduce al tratamiento con pulsos de esteroides, mientras que en la mayoría de los centros no se administra ningún tratamiento cuando se observa esta lesión en las biopsias de seguimiento. En un estudio realizado en más de 500 pacientes monitorizados con biopsias de indicación y de seguimiento, surgieron cambios *borderline* como un grupo diagnóstico heterogéneo, que van desde una inflamación leve y sin consecuencias (resuelta en aproximadamente el 60 % de los casos no tratados) hasta una RMC clínicamente significativa capaz de inducir lesión tubular mediada por el sistema inmunológico [72].

1.2.1.2.4 Lesiones inflamatorias en las zonas de fibrosis intersticial / atrofia tubular.

La FI/AT en biopsias por indicación de trasplante renal es un hallazgo frecuente y se asocia a diferentes enfermedades activas: RMA, RMC, glomerulonefritis, infección por virus del polioma o pielonefritis. Sin embargo, una proporción de las biopsias por indicación y seguimiento solo muestran FI/AT con grados variables de inflamación sin otras lesiones asociadas. Las biopsias de seguimiento que muestran FI/AT con inflamación en áreas no fibróticas (i+ FI/AT) tienen una supervivencia del injerto menor en comparación con las biopsias que solo muestran FI/AT o inflamación [34,38,43,73-75]. Además, la supervivencia del injerto disminuye tanto en biopsias por indicación como en biopsia de seguimiento que presenta inflamación en áreas de fibrosis intersticial (i-FI/AT) [76,77]. Es de destacar que este fenotipo histológico se asocia con la minimización de la inmunosupresión [78]. En conjunto, estos hallazgos han inspirado la definición del RMC crónico activo basándose en la presencia de inflamación en áreas de fibrosis (i-FI/AT ≥2 y t ≥ 2), que se ha incluido en la última clasificación de Banff [26].

1.2.1.3 Diagnóstico molecular de rechazo.

A pesar de los avances en la inmunología del trasplante, hoy en día el RMA sigue siendo una de las principales causas de pérdida del injerto renal. La dicotomía que plantea la clasificación de Banff, reconociendo dos principales fenotipos de rechazo, no es suficiente para interpretar la heterogeneidad de los mecanismos que conducen al rechazo, siendo una limitación la necesidad de obtener muestras representativas del injerto, la baja reproducibilidad interindividual de las lesiones y una falta de diagnóstico certero por ausencia de conocimientos de la evolución cambiante de la clasificación. El análisis de la expresión génica en biopsias de injerto renal (transcriptómica basada en biopsias), ha proporcionado nuevos conocimientos sobre la fisiopatología del rechazo y ha mostrado perfiles de expresión génica que permiten precisar el diagnóstico de RMC y RMA así como otras patologías que pueden estar presentes a nivel del injerto renal.

Sarwal et al., en el 2003, fueron los primeros en realizar micromatrices en biopsias con rechazo, confirmando la expresión de transcritos de células T [79]. En 2004, Flechner et al. [80], realizaron un trabajo donde se compararon biopsias de RMC y cambios BL, ellos pudieron diferenciar las biopsias normales de las que presentaban rechazo, pero no de otros diagnósticos. Reeve et al [81]. distinguió el rechazo del no rechazo mediante análisis predictivos de microarrays en biopsias en las que estaban representadas diferentes patologías. En este estudio se observó que el RMC y RMA compartían transcritos, sobre todo los relacionados con IFN-gamma (p. ej., CXCL9, CXCL11, GPB1), dado que los dos rechazos conducen a la liberación de IFN-gamma: RMC mediante los linfocitos T y el RMA mediante las células NK.

El laboratorio liderado por P. Halloran ha realizado análisis moleculares de biopsias de trasplantes para complementar el diagnóstico histológico convencional mediante la utilización del Sistema de Diagnóstico Microscópico Molecular (MMDx), que emplea algoritmos informáticos basados en la expresión génica para investigar el rechazo y otras lesiones en las biopsias renales. De estas investigaciones se desarrollaron listas de conjuntos de genes llamados conjuntos de transcritos basados en patogénesis (PBT). Estos patrones incluyen la expresión de transcritos inducidos por células T, macrófagos e IFN-gamma, así como transcritos que reflejan la respuesta reparativa [82,83]. Los PBT no describen diagnósticos histológicos, pero dan información sobre las vías biológicas involucradas [84].

En el estudio de Sellarés et al.[85], se obtuvieron los resultados de 403 biopsias que sirvieron como referencia para diseñar los clasificadores moleculares diagnósticos del RMA; lo que permitió distinguir entre inflamación microvascular y fibrosis. El estudio INTERCOM validó estas clasificaciones y evaluó su impacto en el ámbito clínico. En 2017, se llevó a cabo el ensayo INTERCOMEX, una extensión del INTERCOM, con el fin de evaluar la utilidad de la técnica de MMDx en la evaluación de biopsias con diagnósticos de RMC y RMA. El estudio respaldó la viabilidad y los beneficios de utilizar la técnica MMDx en tiempo real en la práctica clínica diaria [86].

En relación con los cambios BL, en el año 2012 de Freitas et al. [87], realizaron un estudio donde se compararon biopsias con cambios BL, biopsias con RMC y biopsias sin rechazo. Observaron que un tercio de los casos diagnosticados como cambios BL por la histología, realmente correspondían a RMC a nivel molecular y los dos tercios restantes correspondían a biopsias normales. Otros estudios sugieren que los cambios BL presentan una considerable variabilidad, desde una inflamación mínima hasta un diagnóstico que resulta en rechazo crónico **[72]**.

Scherer et al.[88], analizaron la expresión génica en biopsias de seguimiento realizadas a los tres meses en pacientes sin fibrosis, contrastándola con aquellos cuyas biopsias, a los seis meses, revelaban ausencia de fibrosis o fibrosis leve. Se concluyó que los pacientes que evidenciaban progresión de fibrosis entre los tres y seis meses, tenían una mayor expresión inicial de transcritos relacionados con la activación de linfocitos T y B, así como con procesos asociados a la fibrosis.

Lo más reciente realizado por el Grupo de Banff ha sido la descripción de un panel de genes para analizar tejido de trasplante de órganos sólidos, incluidos riñones, pulmones, corazón e hígado (Banff Human Organ Transplant, B-HOT), mediante la plataforma Nanostring. Los genes se clasificaron según las respuestas de los receptores al rechazo, la tolerancia, la toxicidad de los fármacos e infecciones virales (758 genes en diagnósticos patológicos y genes de 12 controles sanos) [27]. Varol et al. [89], han realizado un estudio en donde se evaluó la viabilidad del análisis transcriptómico utilizando la técnica Nanostring para poder clasificar entre biopsias con RMA, RMC y/o BL y ausencia de rechazo, para esto se analizaron 96 biopsias de indicación, observaron un patrón de expresión génica distinto en las biopsias BL y/o RMC y RMA en comparación con las biopsias sin rechazo.

Tabla 2. Comparación de técnicas de análisis de expresión de genes utilizadas en transcriptómica (traducido) [90].

Técnica	Tipo de Muestra	Nº máximo de transcritos	Tiempo	Costo	Ventajas	Desventajas	Validado en trasplante
RT-qPCR	Generalmente requiere RNA-later o tejido congelado	Limitado. Depende de la cantidad de RNA	<8 h	Bajo	Económico, fácilmente personalizable	Los genes deben preseleccionarse, requiere paso de amplificación, no apto para RNA degradado	No
RT-MLPA	Apto para FFPE	60	<24 h	Bajo	Económico, fácilmente personalizable	Los genes deben preseleccionarse	No
Microarray	Generalmente requiere RNA-later o tejido congelado	~ 47.000	48 h	Alto	Gran selección de genes, adecuado para estudios de descubrimiento	Requiere un núcleo separado de tejidos, no apto para FFPE requieren envío a un laboratorio central	Sí (MMDx)
Nanostring	Apto para FFPE	800	24-48 h	Intermedio	Adecuado para muestras después del procesamiento histológico rutinario o FFPE archivado	Los genes deben preseleccionarse, acceso a la plataforma Nanostring requerido	No para trasplante (Sí en cáncer)
RNA-Seq	Generalmente requiere RNA-later o tejido congelado	n/a (ARN codificante y no codificante)	Hasta 1 semana	Alto	Secuenciación del genoma completo, amplio rango dinámico; adecuado para estudios de descubrimiento	Costoso, requiere RNA de buena calidad y preparación de bibliotecas, análisis de datos consume tiempo	No

FFPE: fijado con formaldehído y fijado en parafina. RNA-Seq: Secuenciación del transcriptoma entera, RT-MLPA: amplificación de sonda dependiente de ligadura múltiple con transcriptasa inversa, RT-qPCR: reacción en cadena de la polimerasa cuantitativa con transcriptasa inversa.

1.2.2 Monitorización farmacológica.

Entre los años 70 y 90, el tratamiento inmunosupresor presentó avances debido a una mejor comprensión de los mecanismos celulares implicados en el rechazo del injerto. Los linfocitos T desempeñan un papel clave en los eventos inmunológicos iniciales tras la exposición al injerto. La activación y la proliferación de estas células requieren al menos tres señales, las cuales se generan a través de la interacción con los aloantígenos (figura 6) [91].

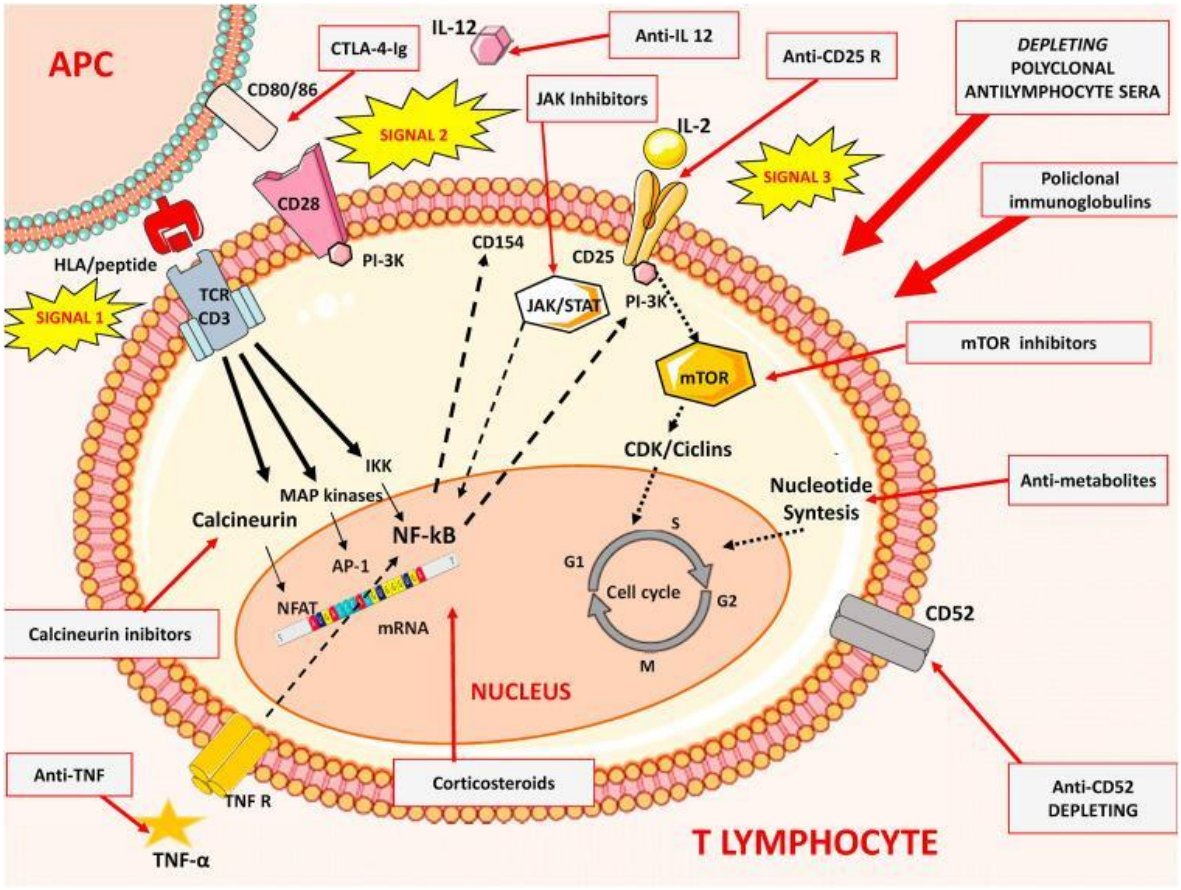

Figura 6. Dianas celulares de células T y células presentadoras de antígenos y sus interacciones y agentes inmunosupresores [91].

La tríada de la inmunosupresión se basa en el régimen de TAC, corticoides y un tercer fármaco que corresponde a un antimetabolito o un i-mTOR. Aunque en la práctica clínica se evalúa de manera rutinaria la monitorización de niveles plasmáticos de los fármacos inmunosupresores (TAC, CsA, SRL, EVE), no se ha establecido la utilidad de la monitorización de los niveles de fármacos AMF [92].

Una limitación importante del fármaco tacrolimus es que presenta una ventana terapéutica estrecha y los niveles séricos óptimos durante su seguimiento (TAC-C_0), no están definidos. La baja exposición a TAC durante el primer año se relaciona con

un mayor riesgo de rechazo clínico y/o subclínico así como un mayor riesgo de desarrollo de ADEs [93]. Diversos estudios han observado una relación entre una menor exposición a TAC y una menor supervivencia del injerto a largo plazo. Por otro lado, una mayor exposición a TAC se ha relacionado con efectos adversos como nefrotoxicidad, infecciones virales y cáncer, entre otros [94,95.] Además, el TAC es un fármaco con baja biodisponibilidad (20-30 %) y su tasa de metabolismo está relacionada con diferentes genes [96].

Para explorar la asociación entre el metabolismo de TAC y los resultados clínicos, se han desarrollado diferentes modelos farmacocinéticos y farmacodinámicos, teniendo en cuenta variables clínicas como el hematocrito, la albúmina sérica, la edad, el sexo o el índice de masa corporal y polimorfismos de los genes más relevantes que codifican las enzimas del citocromo P450 (CYP3A4 y CYP3A5) [97].

En los últimos años, se evaluaron marcadores predictivos que podrían ayudar a estimar el metabolismo del TAC de forma individual y el riesgo de toxicidad. Dentro de las herramientas para la monitorización cuantitativa tenemos el Coeficiente de variabilidad de TAC (CV-TAC), que se representa porcentualmente (CV % = (DE/media) $*$ 100)), la determinación del tiempo en rango terapéutico (TRT), así como el tiempo por debajo o por encima del rango terapéutico y la relación concentración/dosis de TAC (C/D; ng/ml/mg) que se calcula como la relación dosis-concentración media en cada momento. Se ha demostrado que esta herramienta simple y de bajo costo, pero efectiva, podría ayudar no solo a predecir el riesgo individual de toxicidad por TAC, sino también a predecir la función del injerto, el riesgo de rechazo o incluso la supervivencia del paciente.

La variabilidad de los niveles TAC durante el seguimiento postrasplante se relaciona con niveles bajos del fármaco, la falta de adherencia y consecuentemente mayor riesgo de rechazo [98-101]. La alta variabilidad en los niveles de TAC intra e interindividual se asocia con fibrosis intersticial/atrofia tubular (FI/AT) [98], rechazo y fracaso del injerto. Se observó en un estudio, que existe una asociación entre los niveles de TAC inferiores a 5 ng/m y el desarrollo de ADEs; además, se demostró una asociación más significativa cuando estos niveles fueron más bajos durante los 6 meses anteriores a la detección de *ADEs dn* en comparación con períodos anteriores [101].

El tiempo en rango terapéutico (TRT), comúnmente utilizado en el tratamiento anticoagulante, es un concepto relativamente nuevo en el ámbito del trasplante [102].

Este concepto, que calcula el porcentaje de tiempo que un nivel del fármaco se mantiene dentro del rango objetivo predefinido para cada paciente, se ha aplicado a la monitorización con TAC [98]. Basado en los niveles objetivos durante el primer año después del trasplante, un tiempo en rango terapéutico inferior al 60 % se asocia con la aparición de *ADEs dn* y un mayor riesgo de rechazo a los 12 meses, así como con la pérdida del aloinjerto a los 5 años [99].

El uso de estas sencillas herramientas de seguimiento podría ser una estrategia viable para evaluar la adherencia al tratamiento, optimizar la dosificación y mejorar los resultados.

1.2.3 Monitorización inmunológica.

1.2.3.1. Monitorización de Anticuerpos anti-HLA y desajuste de epítopos.

La monitorización inmunológica se realiza mediante la determinación de anticuerpos anti-HLA. Un método común y rápido, menos sensible, pero más específico, es la prueba de citotoxicidad dependiente del complemento (CDC) desarrollada en los años 60. La técnica consiste en hacer reaccionar el suero del receptor con los linfocitos del potencial donante para determinar si existe una reacción antígeno-anticuerpo, capaz de desencadenar una reacción de citotoxicidad dependiente del complemento. La prueba de CDC se emplea tanto para realizar la prueba cruzada pretrasplante como para determinar si el suero de un posible receptor contiene anticuerpos contra los antígenos HLA. Para ello, se utiliza un panel amplio de células que representan la distribución de posibles donantes renales. De este procedimiento surge el concepto de Panel Reactive Antibodies (PRA), que se refiere al porcentaje de células frente a las cuales la prueba cruzada resulta positiva.

En la década de los 80, se implementó la citometría de flujo (CF) lo que aumentó la sensibilidad para detectar anticuerpos anti-HLA debido al uso de anticuerpos monoclonales (anti-IgG) marcados con fluorocromos. Esta técnica permite distinguir los anticuerpos anti-HLA clase II, que se unen únicamente con los linfocitos B, de los anti HLA clase I que reaccionan con linfocitos T y los B.

En los años 90, surgieron los primeros ensayos en fase sólida que permitían solubilizar e inmovilizar moléculas HLA en un soporte sólido [103]. A partir de 2003, la tecnología Luminex® ha revolucionado el campo de la histocompatibilidad [104.] Esta metodología emplea moléculas HLA purificadas de líneas celulares linfoblásticas y las inmoviliza en microesferas con una combinación específica de fluorocromos. Es una técnica semicuantitativa que correlaciona la cantidad de anticuerpos presentes con la cantidad de fluorescencia emitida. Los resultados se expresan mediante un valor numérico conocido como intensidad mediana de fluorescencia (MFI: *median fluorescence intensity)*.

La incidencia de *ADEs dn* puede estar relacionada con el riesgo inmunológico del receptor, el tipo de tratamiento inmunosupresor, la adherencia al tratamiento, el MFI y los antígenos conocidos del donante. Se considera de alto riesgo la presencia de anticuerpos anti-HLA con niveles de MFI superiores a 3000; sin embargo, niveles altos de MFI no siempre indican un título alto de anticuerpos, por lo que la dilución del suero parece ser la estrategia más confiable para determinar un MFI real [105]. La incidencia

de *ADEs dn*, en pacientes de bajo riesgo inmunológico y con triple terapia, es del 2 al 10 %, siendo habitualmente de clase II, especialmente DQ [106]. Se han asociado a una peor supervivencia del injerto aquellos pacientes que presentan ADEs que fijan complemento (fracción C1q del complemento); además, aquellos con MFI más elevados presentan mayor capacidad de fijar complemento [107,108]. La patogenicidad de los anticuerpos también se ve influenciada por el tipo de inmunoglobulina. Estudios previos sobre la incidencia de *ADEs dn* indican que los subtipos IgG1 e IgG3 están vinculados a una menor supervivencia del injerto [109-111].

El anticuerpo anti-HLA también puede estar presente antes del trasplante (ADE preexistente) o desarrollarse después del trasplante en el contexto de infra inmunosupresión [112], a esto se suma además las incompatibilidades HLA [113]. Varios estudios han sugerido que en los casos en donde se observan *ADEs dn* la supervivencia del aloinjerto es inferior en comparación con el rechazo con ADEs preexistentes. La supervivencia del aloinjerto fue del 63 % en pacientes con ADEs preexistente y únicamente del 34 % en pacientes con *ADEs dn* [114]. Sobre la base de la fuerte relación entre *ADEs dn*, rechazo y pérdida del injerto, los pacientes trasplantados con *ADEs dn* deben someterse a una estrecha monitorización de la función del aloinjerto [115].

Se ha descrito que la incompatibilidad HLA donante receptor a nivel de epítopos ofrece más información sobre el riesgo de desarrollo de *ADEs dn* respecto a la incompatibilidad a nivel alélico [116-118]. La aplicación más reciente de esta incompatibilidad de epítopos monomoleculares de HLA-DR/DQ, ha permitido una mejor correlación con *ADEs dn* permitiendo estratificar los receptores en categorías de bajo, intermedio y alto riesgo inmunológico [119].

1.2.3.2 Otros biomarcadores de monitorización inmunológica (sangre y orina).

1.2.3.2.1 ADN libre circulante derivado del donante.

La mayor parte del ADN se encuentra dentro de las células. Sin embargo, una pequeña cantidad de ADN en forma de fragmentos circula de forma libre. Dado que la liberación de ADN del donante en la sangre del receptor es secundaria al daño celular en el injerto, estas moléculas pueden ser biomarcadores del aloinjerto. Lo et al. [120], fueron los primeros en informar sobre la presencia de ADN libre derivado de donantes (dd-cfDNA) en el plasma de los receptores de trasplantes. Recientemente, el estudio ADMIRAL evaluó a 1.094 receptores de trasplantes de riñón y los siguió durante 3 años. Los resultados revelaron que los niveles de dd-cfDNA (Allosure) eran significativamente más altos en pacientes con rechazo clínico y subclínico que en pacientes sin rechazo. El nivel medio de dd-cfDNA fue del 0,23 % en ausencia de rechazo y del 1,6 %en presencia de rechazo (p <0,001) [121]. El VPP para el rechazo fue solo del 59 % y el VPN fue mejor con un 87 % cuando el porcentaje umbral de dd-cfDNA fue del 1 % (la sensibilidad fue del 64 % mientras que la especificidad fue del 73 %). Gielis et al. [122], realizaron un estudio, en donde el aumento del dd-cfDNA por encima de un valor inicial de referencia del 0,8 % se asoció al rechazo agudo, pero también con pielonefritis aguda y necrosis tubular aguda. En este estudio, el 18 % de los aumentos en la fracción dd-cfDNA podría explicarse por la presencia de uno de estos eventos adversos.

1.2.3.2.2 Microarrays en sangre periférica.

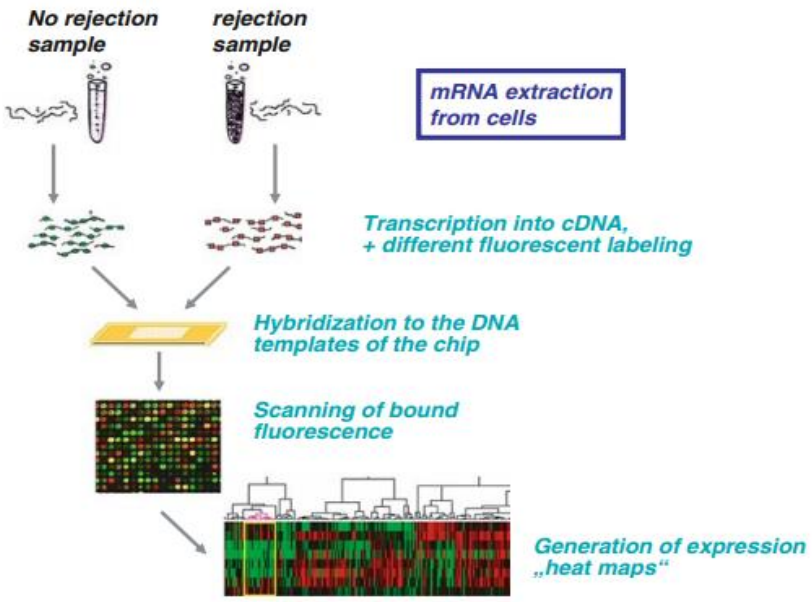

Figura 7. Análisis de expresión de ARNm mediante microarrays de ADN se muestra un ejemplo de análisis diferencial de muestras con y sin rechazo [123].

Los primeros estudios de microarrays se enfocaron en identificar aquellos genes relacionados con rechazo. En un estudio en donde se analizaron 148 muestras pareadas de biopsias renales y de sangre periférica, se observó que 46 de estas muestras correspondían a un diagnóstico histológico normal, 63 a rechazo agudo y 39 a disfunción aguda del injerto, pero sin evidencia de rechazo. La muestra fue dividida en una cohorte exploratoria y otra de validación. Este estudio confirmó la validez de un conjunto de 200 genes que detectan los genes de rechazo más diferencialmente expresados (TruGraf®) [124].

AlloMap ® Kidney (CareDx, Brisbane, CA) utiliza secuenciación de próxima generación y tecnología de secuenciación de ARN dirigida para elaborar perfiles de expresión genética para evaluar la inactividad inmune [125]. Un estudio que evaluó 235 muestras comparadas con resultados histológicos (66 con rechazo y 169 sin rechazo) de 222 pacientes mostró un VPN del 87 % al 95 %. Sin embargo, el VPP para el rechazo del aloinjerto fue solo del 18 % al 40 %, con una sensibilidad del 70 % y una especificidad del 66 % [126].

Otro estudio identificó 17 genes específicos de rechazo agudo y a este conjunto de genes se le denominó 'kidney solid organ response test' (kSORT), aunque la prueba pudo predecir el rechazo con alta sensibilidad y especificidad, no pudo distinguir entre el RMC del RMA [127]. En 2017, Crespo et al. [128], examinaron la capacidad predictiva de la prueba kSORT y del ensayo ELISPOT en 75 pacientes con trasplante renal, realizándoles una biopsia de seguimiento a los 6 meses. Concluyeron que la combinación de ambas técnicas era necesaria para aumentar la capacidad de predecir a pacientes con un alto riesgo de desarrollar un RSC.

A pesar de los estudios sobre genes en sangre periférica para detectar rechazo, los resultados inconsistentes limitan su uso generalizado.

1.2.3.2.3 Biomarcadores en orina (quimiocinas y ARN urinario)

Las quimiocinas CXCR3, CXCL9 y CXCL10 se considera biomarcadores de interés. Resultados de varios estudios de un mismo centro mostraron una elevada correlación entre CXCL9 urinario y RMC y otros entre CXCL10 y RMA [129,130]. El ensayo multicéntrico CTOT-01, demostró que los niveles urinarios de ARNm y proteína de CXCL9 son capaces de diagnosticar con mayor precisión el rechazo agudo, con niveles que se elevan hasta 30 días antes de la detección clínica. Además, los niveles bajos de proteína CXCL9 urinaria a los 6 meses se asociaron con una baja probabilidad de desarrollo futuro de rechazo agudo o reducción de la TFG hasta 2

años después del trasplante[131.] En un estudio liderado por Raban et al.[132], demostraron que la combinación de CXCL10 urinario, junto con la creatinina urinaria y los niveles de ADEs, eran mejores predictores de RMA que la determinación sola de ADEs.

Suthanthiran et al. [133], analizaron el ARNm urinario recolectado en serie de 485 receptores de riñón durante el primer año posterior al trasplante. Se derivó una firma de tres genes utilizando CD3ε, CXCL10 y ARN ribosómico 18S que discriminó el RMC de ausencia de rechazo. El estudio de muestras de orina en pacientes con rechazo reveló una elevada expresión de genes de quimiocinas 120 días antes de la realización de la biopsia.

2. HIPÓTESIS

En la práctica clínica habitual, durante el seguimiento del postrasplante renal, se realiza la monitorización del injerto mediante la determinación de la creatinina sérica, la proteinuria y los anticuerpos HLA. El valor añadido, implementado en algunos centros, de la monitorización histológica a través de biopsias de seguimiento, ha posibilitado la detección precoz de rechazo subclínico y/o cambios *borderline*, que de otra manera pasarían inadvertidos, y que permiten conocer la historia natural de la enfermedad en trasplante renal.

Las hipótesis de este trabajo son:

- La identificación de los genes relacionados con el rechazo en biopsias de seguimiento que muestran cambios *borderline*, fibrosis intersticial y atrofia tubular y rechazo subclínico, podría ser una herramienta eficaz para estratificar el riesgo de pérdida del injerto y adecuar el tratamiento inmunosupresor.

- La disminución de la variabilidad intraindividual de los niveles de tacrolimus durante el seguimiento postrasplante podría ofrecer un control más preciso de la inmunosupresión, lo que disminuiría la susceptibilidad de desarrollo de inflamación subclínica.

- La exposición a los niveles de tacrolimus puede influir en la expresión de los genes relacionados con el rechazo, lo que sugiere que una monitorización farmacológica adecuada podría contribuir a disminuir el riesgo de rechazo subclínico y cambios *borderline* para mejorar la supervivencia del injerto

3. OBJETIVOS

3.1 Objetivo principal.

Caracterizar la asociación entre la exposición a tacrolimus, la inflamación subclínica, la progresión de la fibrosis intersticial y atrofia tubular (FI/AT) y la expresión de genes asociados al rechazo en biopsias de seguimiento en trasplante renal, así como su impacto en el pronóstico del injerto.

3.2 Objetivos secundarios.

- Caracterizar la asociación entre la expresión génica y el diagnóstico histológico (histología normal, fibrosis, inflamación e inflamación en áreas de fibrosis) en biopsias de seguimiento y por indicación clínica.

- Determinar la asociación entre las lesiones histológicas y la exposición a tacrolimus y su metabolismo en biopsias de seguimiento pareadas.

- Investigar la asociación entre los niveles de tacrolimus y la expresión de genes de rechazo y su relación con la inflamación subclínica y evolución del injerto renal a medio plazo.

4. COMPENDIO DE PUBLICACIONES

Artículo 1

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4.1 A Rejection Gene Expression Score in Indication and Surveillance Biopsies Is Associated with Graft Outcome.

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A Rejection Gene Expression Score in Indication and Surveillance Biopsies Is Associated with Graft Outcome

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Abstract: Rejection-associated gene expression has been characterized in renal allograft biopsies for cause. The aim is to evaluate rejection gene expression in subclinical rejection and in biopsies with borderline changes or interstitial fibrosis and tubular atrophy (IFTA). We included 96 biopsies. Most differentially expressed genes between normal surveillance biopsies ($n = 17$) and clinical rejection ($n = 12$) were obtained. A rejection-associated gene (RAG) score was defined as its geometric mean. The following groups were considered: (a) subclinical rejection (REJ-S, $n = 6$); (b) borderline changes in biopsies for cause (BL-C, $n = 13$); (c) borderline changes in surveillance biopsies (BL-S, $n = 12$); (d) IFTA in biopsies for cause (IFTA-C, $n = 20$); and (e) IFTA in surveillance biopsies (IFTA-S, $n = 16$). The outcome variable was death-censored graft loss or glomerular filtration rate decline ≥ 30 % at 2 years. A RAG score containing 109 genes derived from normal and clinical rejection (area under the curve, AUC = 1) was employed to classify the study groups. A positive RAG score was observed in 83% REJ-S, 38% BL-C, 17% BL-S, 25% IFTA-C, and 5% IFTA-S. A positive RAG score was an independent predictor of graft outcome from histological diagnosis (hazard ratio: 3.5 and 95% confidence interval: 1.1–10.9; $p = 0.031$). A positive RAG score predicts graft outcome in surveillance and for cause biopsies with a less severe phenotype than clinical rejection.

Keywords: renal transplantation; biopsies; rejection; transcriptomics; microarrays; borderline changes; interstitial fibrosis and tubular atrophy

1. Introduction

Rejection-associated gene expression has been well-characterized in for cause renal allograft biopsies. Three groups of rejection gene transcripts have been differentiated: (a) gene transcripts that are shared by T cell-mediated and antibody-mediated rejection; (b) specific differentially expressed transcripts in T cell-mediated rejection; and (c) specific differentially expressed transcripts in

antibody-mediated rejection [1–3]. Rejection-associated gene expression is shared among different transplanted organs [4] and is involved in the immune response against infection, cancer, or autoimmune diseases [5]. They are also expressed in surveillance biopsies with subclinical rejection [6], and in biopsies with a histological diagnosis below the threshold of rejection such as borderline changes or interstitial fibrosis and tubular atrophy (IFTA).

Subclinical rejection in surveillance biopsies has been associated in some studies with decreased renal allograft survival [7–9]. It has been shown in a study comparing clinical and subclinical rejection that both phenotypes shared differentially expressed genes and pathways. These data suggest that there exists a continuum of alloimmune activation in both situations [10].

The significance of borderline changes is difficult to interpret since it can represent true T cell-mediated rejection (TCMR), acute kidney injury associated with ischemia-reperfusion or other types of tissue injury [11]. Thus, it is not surprising that the histological definition of borderline changes has been modified over the years. In the first Banff meeting, this category was defined as the presence of mild tubulitis (t1) associated with mild to severe interstitial inflammation (i1–i3) [12]. Later, the classification was modified, and biopsies showing mild to severe tubulitis (t1–t3) without interstitial inflammation (i0) were included in this category [13]. Recently, it has been described that patients with biopsy scoring ≥ i1t1 have a poorer outcome than patients with biopsies displaying i0t1 [14], leading to the actual definition of borderline changes as at least i1t1 [15]. Therapeutic approaches towards borderline changes vary in different clinical settings. The diagnosis of borderline changes in biopsies for cause leads to anti-rejection treatment with steroid pulses in most cases, while in many centers the presence of borderline changes in surveillance biopsies is not treated. Thus, borderline changes are considered a heterogeneous diagnosis, ranging from mild inconsequential inflammation that is resolved without a specific treatment to full blown TCMR [16–18]. Microarray studies in biopsies for cause displaying borderline changes have shown that most cases designated borderline by histopathology are found to be non-rejection by molecular phenotyping [11]. In the setting of surveillance biopsies, it has been shown that molecular changes of rejection are correlated with histological diagnosis of TCMR or borderline rejection, but this molecular pattern is not associated with graft outcomes [19].

The presence of IFTA in biopsies for cause has been described in association with antibody-mediated rejection, T cell-mediated rejection, glomerulonephritis, and other diseases affecting the graft. On the other hand, IFTA without the presence of a well-defined post-transplant disease is observed in a proportion of patients [20,21]. In surveillance biopsies, IFTA with interstitial inflammation in healthy areas is associated with decreased graft survival when compared with biopsies with IFTA without inflammation [8].

Microarray studies in surveillance biopsies with IFTA have described the presence of rejection-associated transcripts [7]. These observations inspired the definition of chronic T cell-mediated rejection as the presence of moderate to severe inflammation in areas of fibrosis (i-IFTA ≥ 2 and t ≥ 2) [22].

The aim of the present study is to characterize rejection-associated gene transcripts in for cause and surveillance biopsies with subclinical rejection, borderline changes and IFTA.

2. Results

2.1. Patients

Between July 2015 and August 2018, 435 renal graft biopsies were performed, and 188 biopsies had a third core of tissue stored in the nephrology biobank for microarray studies. Since in 7 patients extracted RNA was of insufficient quality for analysis, 181 patients were considered. Patients with the following histological diagnosis were discarded: chronic pyelonephritis (1), focal and segmental glomerular sclerosis (5), membranous nephropathy (2), IgA nephropathy (6), C3 glomerulopathy (1), polyoma virus BK nephropathy (4) and diabetic nephropathy (1). Histological diagnosis of the considered 161 biopsies is shown in Table 1. Since rejection was an infrequent diagnosis, all cases were

included in the microarray study. For the remaining groups, biopsies with the longest follow up were included to complete a total of 96 biopsies (Table 1). Clinical characteristics of the included patients and histological scores are summarized in Tables 2 and 3.

2.2. Principal Component Analysis Using Gene Transcripts

The principal component analysis showed that rejection in indication and surveillance biopsies clustered in the left upper quadrant, normal surveillance biopsies in the lower right quadrant, while borderline changes and IFTA tended to cluster in between (Figure 1).

Figure 1. Principal component analysis of the microarray and histological diagnosis in for cause and surveillance biopsies. REJ-C, rejection in biopsies for cause ($n = 12$); Normal-S, normal surveillance biopsies ($n = 17$); REJ-S, rejection in surveillance biopsies ($n = 6$); BL-C, borderline changes in biopsies for cause ($n = 13$); BL-S, borderline changes in surveillance biopsies ($n = 12$); IFTA-C, interstitial fibrosis and tubular atrophy in biopsies for cause ($n = 20$); IFTA-S, interstitial fibrosis and tubular atrophy in surveillance biopsies ($n = 16$).

2.3. Rejection-Associated Gene Score (RAG Score)

We described the most differentially expressed genes between biopsies for cause with clinical rejection and normal surveillance biopsies. For this purpose, we adjusted FC and p-value to obtain approximately the 100 most differentially expressed genes between these two groups. A total of 109 differentially expressed genes (p-value < 0.01 and fold change (FC) $>$ log 1.75) were obtained as listed in Supplement Table S1. The geometric mean of these 109 differentially expressed genes was calculated to obtain a RAG score.

The RAG score was similar between rejection in biopsies for cause (REJ-C) and subclinical rejection (REJ-S). In borderline changes in biopsies for cause (BL-C), borderline changes in surveillance biopsies (BL-S), interstitial fibrosis and tubular atrophy in biopsies for cause (IFTA-C), and IFTA in surveillance biopsies (IFTA-S) the score was significantly lower than in rejection biopsies. On the other hand, it was significantly higher in BL-C, BL-S, and IFTA-C, than in normal biopsies. There were no differences in the RAG score between IFTA-S and, normal surveillance biopsies (Normal-S) (Figure 2).

To validate this result, we considered the T cell-mediated rejection most differentially expressed genes in kidney transplants as reported by Venner et al. [23], the common rejection module reported by Khatri et al. [4] and the constant of rejection reported by Wang et al. [5]. These three gene sets were identified in our microarray and a rejection score for each of these three gene sets was calculated as the geometrical mean in our sample. We observed that rejection genes described in these studies were significantly higher in rejection than in the other groups. Using the rejection scores obtained from these three studies, we observed that rejection genes in BL-C were significantly higher than in normal biopsies. The rejection scores obtained from Wang et al. and Khatri et al., were significantly higher in BL-C than in IFTA-S. However, there was no difference in the expression of rejection gene score between BL-C, BL-S, and IFTA-C (Figure 3).

Table 1. Available and included for cause and surveillance biopsies.

	Available Biopsies		Included Biopsies	
	For Cause	Surveillance	For Cause	Surveillance
Normal	0	39	0	17
Borderline changes	19	18	13	12
IFTA	35	32	20	16
TCMR	6	5	6 [a]	5 [c]
ABMR	6	1	6 [b]	1 [d]
Total	66	95	45	51

IFTA, interstitial fibrosis and tubular atrophy; TCMR, T cell-mediated rejection; ABMR, antibody-mediated rejection. [a] 3 cases of TCMR grade I and 3 cases grade II; [b] 2 cases of act ABMR and 4 cases of chronic active ABMR; [c] 2 cases of TCMR grade I and 3 cases grade II and [d] 1 case of active ABMR.

Table 2. Demographic data for recipients and donors as well as clinical data at the time of biopsy.

Variable	Normal	REJ-C	REJ-S	BL-C	BL-S	IFTA-C	IFTA-S	*p*-Value
N	17	12	6	13	12	20	16	
Age (y)	54 ± 13	43 ± 12	53 ± 21	59 ± 11 [b]	57 ± 13 [b,c]	46 ± 15 [c]	52 ± 17	0.060
Sex (m/f)	12/5	6/6	4/2	8/5	9/3	16/4	10/6	0.610
1st Tx/Re-Tx	17/0	8/4	6/0	13/0	12/0	18/2	12/4	0.002
Donor age (y)	56 ± 15	44 ± 16	59 ± 15	59 ± 14	53 ± 16	58 ± 11	57 ± 16	0.226
HLA (A + B + DR) mm	3.8 ± 1.0	3.7 ± 1.4	4.0 ± 1.9	3.9 ± 1.0	3.8 ± 0.7	3.5 ± 1.3	3.7 ± 1.1	0.942
DGF (no/yes)	15/2	10/1	6/0	5/8	10/2	14/1	13/3	0.010
Rejection (no/yes)	17/0	8/4	4/2	12/1	9/3	19/1	14/2	0.082
Induction (ATG/IL2RAb)	4/13	7/5	2/4	4/15	7/9	4/14	7/9	0.295
Immunosuppression TAC + MMF + S/other	16/1	10/2	4/2	9/4	12/0	14/6	16/0	0.036
Time biopsy (m)	6 ± 5	51 ± 57 [a]	6 ± 6 [b]	25 ± 27	7 ± 4 [b,d]	77 ± 78 [a,c,e]	7 ± 5 [b,f]	<0.001
Creatinine (mg/dL)	1.3 ± 0.3	2.0 ± 0.6 [a]	1.4 ± 0.2	2.5 ± 0.8 [a,b,c]	1.3 ± 0.3 [b,d]	2.1 ± 0.7 [a,c,e]	1.4 ± 0.3 [b,d,f]	<0.001
UPCR (g/g)	0.4 ± 0.3	1.7 ± 1.5 [a]	0.3 ± 0.1 [b]	1.1 ± 1.2 [a,b,c]	0.3 ± 0.2 [b,d]	1.3 ± 0.9 [a,c,e]	0.3 ± 0.3 [b,d,f]	<0.001
DSA (no/yes)	17/0	8/4	5/1	13/0	12/0	20/0	16/0	<0.001

Tx, transplant; HLA mm, HLA mismatches at the loci A + B + DR; DGF, delayed graft function; TAC + MMF + S, tacrolimus associated with mycophenolate and steroids; UPCR, urinary protein to creatinine ratio; DSA, HLA donor specific-antibodies determined by Luminex technology. [a] $p < 0.05$ vs. normal, [b] $p < 0.05$ vs. REJ-C, [c] $p < 0.05$ vs. REJ-S, [d] $p < 0.05$ vs. BL-C, [e] $p < 0.05$ vs. BL-S, [f] $p < 0.05$ vs. IFTA-C by Scheffé test.

Table 3. Histological Banff scores.

Variable	Normal	REJ-C	REJ-S	BL-C	BL-S	IFTA-C	IFTA-S
N	17	12	6	13	12	20	16
Glomeruli (N)	16 ± 8	16 ± 6	17 ± 5	17 ± 6	21 ± 11	17 ± 8	23 ± 12
Gs (%)	7 ± 7	22 ± 23	12 ± 16	22 ± 15	12 ± 14	31 ± 24	10 ± 6
g	0.1 ± 0.3	1.4 ± 1.1	0.8 ± 1.3	0.2 ± 0.4	0.4 ± 0.8	0.3 ± 0.6	0.2 ± 0.4
i	0.1 ± 0.2	1.3 ± 1.1	1.5 ± 0.5	1.2 ± 0.7	0.7 ± 0.6	0.1 ± 0.3	0.1 ± 0.2
t	0	1.2 ± 0.9	1.8 ± 0.7	1.0 ± 0	1.0 ± 0.6	0.2 ± 0.4	0.1 ± 0.3
v	0	0.3 ± 0.6	0.5 ± 0.5	0	0	0	0
ah	0.2 ± 0.4	1.4 ± 1.2	0	1.0 ± 0.9	0.7 ± 0.-6	1.3 ± 1.2	0.6 ± 0.6
cg	0	0.7 ± 0.9	0	0	0.2 ± 0.6	0.2 ± 0.5	0
ci	0.1 ± 0.3	1.2 ± 0.9	0.8 ± 1.0	1.3 ± 0.6	0.9 ± 0.8	1.8 ± 0.8	1.3 ± 0.5
ct	0.6 ± 0.5	1.1 ± 0.8	1.0 ± 0.6	1.2 ± 0.4	1.0 ± 0.4	1.6 ± 0.9	1.1 ± 0.3
cv	0.4 ± 0.7	0.8 ± 0.8	0.3 ± 0.5	1.0 ± 0.8	0.4 ± 0.5	1.3 ± 1.2	0.9 ± 0.8
mm	0.1 ± 0.2	0.5 ± 0.7	0	0.1 ± 0.3	0	0.2 ± 0.7	0
ptc	0.1 ± 0.2	1.4 ± 0.8	0.7 ± 1.2	0.6 ± 0.8	0.4 ± 0.8	0.5 ± 0.8	0.1 ± 0.2
C4d	0	0.3 ± 0.5	0	0	0	0	0
i-IFTA	0.4 ± 0.9	2.1 ± 1.3	2.2 ± 1.3	2.1 ± 0.8	1.3 ± 1.1	2.2 ± 1.0	1.8 ± 0.9
t-IFTA	0.2 ± 0.4	0.9 ± 0.6	0.8 ± 0.4	0.7 ± 0.7	0.6 ± 0.7	0.7 ± 0.6	0.7 ± 0.5
i-total	0.1 ± 0.2	1.3 ± 0.6	1.7 ± 0.6	1.5 ± 0.7	0.8 ± 0.6	0.7 ± 0.5	0.4 ± 0.3

Gs, percentage of globally sclerosed glomeruli; g, glomerulitis; i, interstitial infiltrate; t, tubulitis; v, endothelialitis; ah, arteriolar hyalinosis; cg, transplant glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, intimal thickening; mm, mesangial matrix increase; C4d, deposition of C4d in peritubular capillaries; i-IFTA, inflammation in areas of interstitial fibrosis; t-IFTA, tubulitis in atrophic tubules.

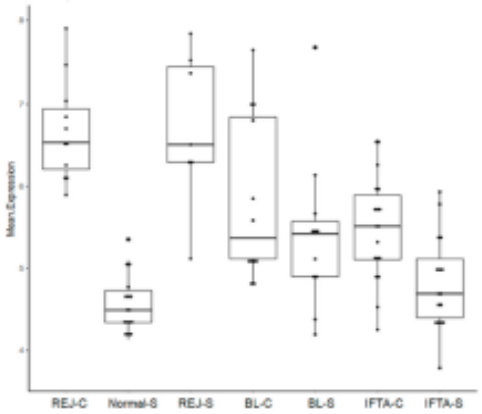

Figure 2. Rejection-associated gene score in the different study groups. REJ-C, rejection in biopsies for cause (n = 12); Normal-S, normal surveillance biopsies (n = 17); REJ-S, rejection in surveillance biopsies (n = 6); BL-C, borderline changes in biopsies for cause (n = 13); BL-S, borderline changes in surveillance biopsies (n = 12); IFTA-C, interstitial fibrosis and tubular atrophy in biopsies for cause (n = 20); IFTA-S, interstitial fibrosis and tubular atrophy in surveillance biopsies (n = 16). ANOVA p-value 6.27×10^{-12}.

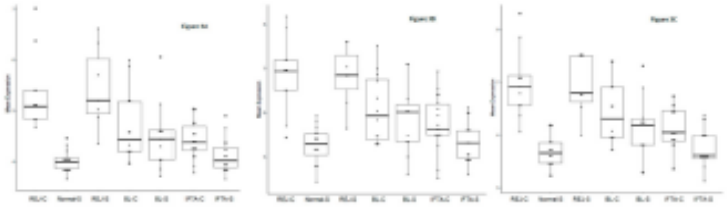

Figure 3. Rejection gene score according to the most differentially expressed genes in Venner et al. [23], Khatri et al. [4], and Wang et al. [5] applied to our set of biopsies ((**A**–**C**) respectively). REJ-C, rejection in biopsies for cause (n = 12); Normal-S, normal surveillance biopsies (n = 17), REJ-S, rejection in surveillance biopsies (n = 6); BL-C, borderline changes in biopsies for cause (n =13); BL-S, borderline changes in surveillance biopsies (n = 12); IFTA-C, interstitial fibrosis and tubular atrophy in biopsies for cause (n = 20); IFTA-S, interstitial fibrosis and tubular atrophy in surveillance biopsies (n = 16). ANOVA p-value 9.46×10^{-10}, 2.16×10^{-9}, 5.08×10^{-10} for graphs (**A**–**C**); respectively.

2.4. RAG Score and Outcome

There was no overlap in the RAG score between REJ-C biopsies and Normal-S biopsies (Figure 2). By receiver operating curve analysis, the best cut off was 5.89 (Youden's index) with an area under the curve of 1. Accordingly, the studied groups of biopsies were classified as positive (≥5.89) or negative (<5.89) RAG score (Figure 4).

In the 5 study groups, graft outcome (death-censored graft loss or 2-year eGFR deterioration ≥ 30%) was associated with histological diagnosis and with RAG score (Figure 5).

Regarding the control groups, none of the Normal-S group and 6 out of 12 patients with REJ-C reached the composite endpoint.

Multivariate Cox regression analysis showed that RAG score ≥ 5.89 was an independent predictor of graft outcome from histological diagnosis (hazard ratio: 3.5 and 95% confidence interval: 1.1–10.9; p = 0.031). Survival analysis excluding patients with subclinical rejection yielded similar results (p = 0.004 for univariate and p = 0.037 for multivariate analysis).

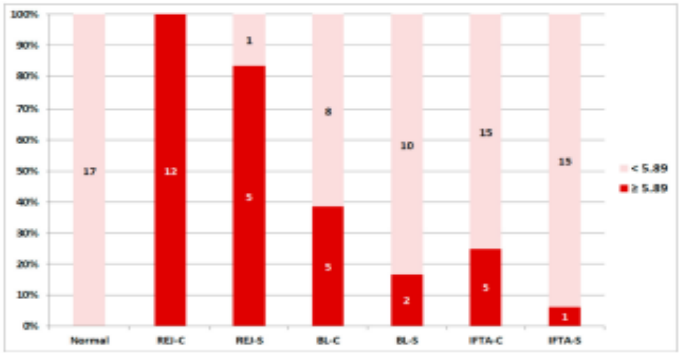

Figure 4. Positive (≥5.89) and negative (<5.89) rejection-associated gene score in the different histological categories in surveillance and for cause biopsies. The number of biopsies in each group according to the rejection-associated gene score is displayed. REJ-C, rejection in biopsies for cause; Normal, normal surveillance biopsies, REJ-S, rejection in surveillance biopsies; BL-C, borderline changes in biopsies for cause; BL-S, borderline changes in surveillance biopsies; IFTA-C, interstitial fibrosis and tubular atrophy in biopsies for cause; IFTA-S, interstitial fibrosis and tubular atrophy in surveillance biopsies.

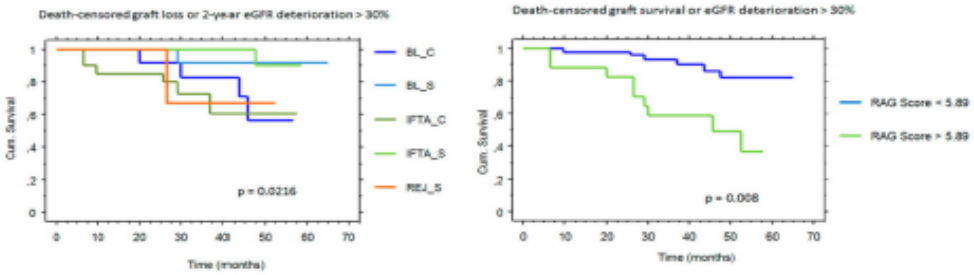

Figure 5. Death-censored graft loss or 2-year eGFR deterioration ≥ 30 % from the date of biopsy in the different histological categories (left panel) and in biopsies with a positive or negative rejection-associated gene (RAG) score (right panel). REJ-S rejection in surveillance biopsies; BL-C, borderline changes in biopsies for cause; BL-S, borderline changes in surveillance biopsies; IFTA-C, interstitial fibrosis and tubular atrophy in biopsies for cause; IFTA-S, interstitial fibrosis and tubular atrophy in surveillance biopsies.

3. Discussion

In the present study, most differentially expressed genes between normal surveillance biopsies and biopsies for cause with rejection, were consistent with rejection-associated gene sets reported by others [4,5,23]. These lists of gene sets have been obtained by different approaches. Venner et al. [23] described the most differentially expressed genes between T cell-mediated rejection and all other diagnoses, including antibody-mediated rejection in biopsies for cause. Khatri et al. [4] employed eight independent data sets from kidney, liver, heart, and lung transplants to characterize the top rejection differentially expressed genes between organs. Wang et al. [5] considered top differentially expressed genes in allograft or cancer rejection, autoimmune disease, and tissue damage during infection. The concordance in the characterization of the top expressed genes during rejection between studies employing different strategies, reflects the stereotyped effector immune response leading to tissue damage triggered by different injuries. In our study, the RAG-score mainly is composed by a set of genes related with antigen presentation, T-cell activation, cytotoxic proteins, chemokine expression, B-cell, and plasma-cell transcripts.

We observed that the RAG-score was not different in surveillance biopsies with subclinical rejection and biopsies for cause with clinical rejection. RAG-score was binarized to classify biopsies as rejection

or non-rejection. Only one out of six patients with rejection in surveillance biopsies had a negative RAG-score. This observation suggests that in subclinical rejection, as it has been previously described in clinical rejection [2], there is a reasonable concordance between histological and molecular diagnosis. Furthermore, this observation argues in favor for treating patients with subclinical rejection [18,24,25]

In biopsies for cause with borderline changes or IFTA, and in surveillance biopsies with borderline changes, RAG-score was higher than in normal surveillance biopsies but lower than in biopsies with clinical rejection. A rejection signal was observed in 83% of surveillance biopsies with rejection, 38% of biopsies for cause with borderline changes, 25% of biopsies for cause with IFTA, 17% of surveillance biopsies with borderline changes and 5% of surveillance biopsies with IFTA. The RAG score variability in these groups suggests that there were patients with and without rejection signal in each diagnostic category. Finally, surveillance biopsies with IFTA were not different from normal surveillance biopsies pointing out that stable grafts with IFTA are immunologically quiescent.

The clinical significance of borderline changes is difficult to interpret ranging from true rejection to non-specific inflammation [16]. Lipman et al. [26] showed that inflammatory gene expression in surveillance biopsies was negative in biopsies with normal histology, intermediate in borderline changes, and high in rejection biopsies. Enhanced inflammatory gene expression in surveillance biopsies with borderline changes and rejection was confirmed in a study evaluating early 6-week surveillance biopsies [19]. However, in this study, enhanced inflammatory gene expression was higher in patients with delayed graft function than in patients with immediate function, suggesting that, in early surveillance biopsies, inflammatory gene expression may also reflect injury-repair response. In the other hand, no association between inflammatory gene expression and 2-year graft outcome was observed. However, studies evaluating the utility of histologic diagnosis in early surveillance biopsies to predict outcome have shown that a long follow up is necessary to show an association between early inflammation and graft events [27–30]. In the present study, RAG-gene score in biopsies with borderline changes was higher in biopsies for cause than in surveillance biopsies. A similar observation was reported in a study comparing early biopsies for cause and 3-month surveillance biopsies [31]. These data suggest that functional deterioration in patients with borderline changes could be partly explained by enhanced rejection associated gene expression. In biopsies for cause with borderline changes, a positive RAG-score, suggests that these cases represent true rejection. This result agrees with previous studies reporting a rejection molecular signature in a similar proportion of cases [11,32].

In patients with IFTA, the RAG-score was positive in one quarter of biopsies for cause and only in 1 out of 20 surveillance biopsies. Graft survival is shortened in surveillance biopsies with IFTA and inflammation in comparison to biopsies with IFTA without inflammation [7,33]. In one-year surveillance biopsies with IFTA and inflammation an overexpression of innate immune transcripts, antigen presentation and cytotoxic T-cell has been described suggesting that mediators of rejection signaling are activated [34]. In another study also evaluating 12-month surveillance biopsies without rejection, biopsies with IFTA expressed macrophage, IFN-gamma, T-cell antigen presentation and T-cell toxicity associated genes [35]. Characterization of differentially expressed genes between normal biopsies and biopsies with IFTA has confirmed that genes related to immune response, inflammation and matrix deposition are overexpressed in biopsies with fibrosis [36]. Furthermore, in a study including for cause and surveillance biopsies, Modena et al. [6] not only described the presence of rejection associated genes in biopsies with IFTA and inflammation, but also in biopsies with IFTA without inflammation. Finally, in another study, it has been observed that there is an overexpression of T cell, IFN-gamma, macrophage and injury-repair transcripts in biopsies with inflammation in healthy areas. By contrast, in biopsies with IFTA and inflammation in scarred areas there is an overexpression of B cells, immunoglobulins, mast cells, and a different set of injury-repair transcripts [37].

In summary, the above-mentioned studies suggest that in patients with IFTA, especially when it is associated with inflammation, there is an over expression of rejection associated genes. In the present study, the inflammatory burden in for cause or surveillance biopsies with IFTA was relatively low. However, a significant proportion of biopsies for cause with IFTA were classified as rejection according

to the RAG-score. Altogether our data confirm that there is a discrepancy between histological diagnosis and gene score [38], especially in patients without a histological diagnosis of clinical rejection, i.e., borderline changes and IFTA.

Finally, a positive RAG-score was associated with graft outcome in the study groups. This association was independent from biopsy indication and histological diagnosis. Interestingly, this association was confirmed when patients with subclinical rejection were excluded from the analysis. The main limitation of the study is the lack of a validation cohort to confirm the utility of RAG-score to predict outcome. However, in another study evaluating the utility of a rejection gene score in patients with IFTA without inflammation, either in for cause or surveillance biopsies, there was an association between a high rejection gene score and graft survival [6]. Another limitation is the reduced sample size, especially to evaluate the utility of the RAG-score in surveillance biopsies.

4. Materials and Methods

4.1. Patients

All adult patients biopsied between July 2015 and August 2018, who gave their informed consent to obtain an additional biopsy core for the nephrology biobank were considered. For the present study, 2 controls and 5 study groups were defined. Control groups were (a) normal surveillance biopsies (Normal-S) and (b) rejection (T cell-mediated and antibody-mediated rejection) in biopsies for cause (REJ-C). The study groups were (a) rejection (T cell-mediated and antibody-mediated rejection) in surveillance biopsies (REJ-S); (b) borderline changes in biopsies for cause (BL-C); (c) borderline changes in surveillance biopsies (BL-S); (d) IFTA in biopsies for cause (IFTA-C); and (e) IFTA in surveillance biopsies (IFTA-S). Clinical T cell-mediated rejection was treated with steroid boluses, active antibody-mediated rejection with plasmapheresis, intravenous immunoglobulins, and rituximab while chronic antibody-mediated rejection was not treated. REJ-S and BL-C were treated according to the attending physician criteria. BL-S and IFTA-C were not treated.

Clinical and demographic characteristics of patients were recorded and anti HLA antibodies at the day of transplant and at the time of biopsy were determined by Luminex technology using the product LIFECODES LifeScreen Deluxe (Gen-Probe-Immucor, Stanford, CT, USA). The present study has been approved by our ethical committee (Comité Etico de Investigación Clínica del Hospital Universitari Vall d'Hebron PR(AG)369/2014, approval date 1 December 2014) and has been performed in accordance with the Declaration of Helsinki, and is consistent with the Principles of the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

4.2. Biopsies

Renal biopsies were performed under ultrasound guidance by trained radiologists with a 16-gauge automated needle. Three cores of tissue were obtained: one was processed for optical microscopy; one was embedded in OCT for immunofluorescence and the other one was stored in RNA later for molecular studies.

The first core was embedded in formalin, paraffin-fixed and 2–4 m thick sections were stained with haematoxylin-eosin, periodic acid Schiff, Masson's trichrome, and silver methenamine. Histological lesions were evaluated according to Banff criteria [15] and accordingly the definition of borderline changes was: $i \geq 1$ and $t \geq 1$. All biopsies were stained with an anti-SV40 antibody. Immunofluorescence studies were done in 3-µm cryostat sections stained with FITC-conjugated anti-human IgG, IgA, IgM, C3, C4, lambda and kappa light chains. C4d was stained with indirect immunofluorescence with a monoclonal antibody (Quidel, San Diego, CA, USA) and deposition in peritubular capillaries was graded according to the Banff criteria. The third score was treated with Ambion® RNAlater® Tissue Collection reagent as indicated by the manufacturer and frozen at −80 °C.

4.3. RNA Extraction and Microarray Hybridization

Total RNA extraction from renal biopsies was performed by lysing the cells with the TissueLyser II and following the RNAeasy Mini Kit (QIAGEN). RNA quantity and quality were analyzed with the NanoDrop ND2000 (Thermo Scientific, Wilmington, DE, USA) and the Bioanalyzer (Agilent Technologies, Sta. Clara, CA, USA).

Microarrays service was carried out by the High Technology Unit (UAT) at Vall d'Hebron Research Institute (VHIR), Barcelona (Spain), using a GeneTitan® System according to the procedure described by the manufacturer. One plate, containing 96 Clariom S arrays, was used for this experiment. These arrays provide an accurate measurement of the human transcriptome at a gene-level, by using probes covering more than 20,000 well-annotated genes, distributed through constitutive exons.

Briefly, 70 ng of total RNA from each sample were used as starting material. The quality of the isolated RNA was measured previously by capillary electrophoresis (Bioanalyzer 2100, Agilent Technologies, Sta. Clara, CA, USA). Single stranded-cDNA suitable for labeling was generated from total RNA using the WT PLUS Reagent Kit (ThermoFisher Scientific, Lutterworth, UK), according to the manufacturer's instructions. Purified sense-strand cDNA was fragmented, labeled, and hybridized to the arrays using the GeneTitan Hybridization, Wash and Stain Kit for WT Plates (ThermoFisher Scientific, Lutterworth, UK). The plate was loaded into the GeneTitan and, after array scanning, raw data quality control was performed to check the performance of the whole processing.

4.4. Statistics

Results are expressed as raw numbers for categorical variables and as the mean ± standard deviation for continuous variables. Comparison between groups for categorical variables was done by chi-squared test with continuity correction. Comparison between groups for continuous variables was done by analysis of variance (ANOVA) with Scheffé post hoc test for individual comparisons. Kaplan–Meier survival curves with log-rank test and Cox's proportional hazard model were employed for survival analysis. A composite outcome variable including death-censored graft loss and eGFR deterioration $\geq 30\%$ at 2-years was defined. All p-values were two-tailed and p-value < 0.05 was considered significant.

Bioinformatic analysis was performed at the Statistics and Bioinformatics Unit (UEB) of the Vall d'Hebron Institute of Research (VHIR, Barcelona, Spain). Robust Multi-array Average (RMA) algorithm [39] was used for pre-processing microarray data. Background adjustment, normalization, and summarization of raw core probe expression values were defined so that the exon level values were averaged to yield one expression value per gene. Selection of differentially expressed genes was based on a linear model analysis with empirical Bayes modification for the variance estimates [40]. To account for multiple testing, p-values were adjusted to obtain stronger control over the false discovery rate (FDR), as described by the Benjamini and Hochberg method [41].

Principal component analysis was performed with normalized data from all the genes used in the differential expression analysis. Differentially expressed genes were selected based on a p-value < 0.01 and base 2 logarithmic fold change > 1.75. In order to validate our rejection-associated gene set, we calculated in our array the geometric mean of the gene expression of the published list of genes described by Venner et al. [23], Khatri et al. [4], and Wang et al. [5].

5. Conclusions

In conclusion, the present data support the utility of the characterization of rejection-associated genes in biopsies with borderline changes, IFTA, and subclinical rejection to improve the risk stratification. These data also raise the question whether characterization of rejection gene expression in biopsies without a clinical and histological diagnosis of rejection may be useful to adjust immunosuppressive treatments and improve outcome.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/21/8237/s1.

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Abbreviations

IFTA	Interstitial fibrosis and tubular atrophy
BL-C	Borderline changes in biopsies for cause
BL-S	Borderline changes in surveillance biopsies
IFTA-C	Interstitial fibrosis and tubular atrophy in biopsies for cause
IFTA-S	Interstitial fibrosis and tubular atrophy in surveillance biopsies
REJ-C	Rejection in biopsies for cause
REJ-S	Rejection in surveillance biopsies
RAG	Rejection-associated gene score

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Artículo 2

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4.2 Progression of Interstitial Fibrosis and Tubular Atrophy in Low Immunological Risk Renal Transplants Monitored by Sequential Surveillance Biopsies: The Influence of TAC Exposure and Metabolism.

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Progression of Interstitial Fibrosis and Tubular Atrophy in Low Immunological Risk Renal Transplants Monitored by Sequential Surveillance Biopsies: The Influence of TAC Exposure and Metabolism

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Abstract: The combination of tacrolimus (TAC) and mycophenolate is the most widely employed maintenance immunosuppression in renal transplants. Different surrogates of tacrolimus exposure or metabolism such as tacrolimus trough levels (TAC-C_0), coefficient of variation of tacrolimus (CV-TAC-C_0), time in therapeutic range (TTR), and tacrolimus concentration dose ratio (C/D) have been associated with graft outcomes. We explore in a cohort of low immunological risk renal transplants ($n = 85$) treated with TAC, mycophenolate mofetil (MMF), and steroids and then monitored by paired surveillance biopsies the association between histological lesions and TAC-C_0 at the time of biopsy as well as CV-TAC-C_0, TTR, and C/D during follow up. Interstitial inflammation (i-Banff score ≥ 1) in the first surveillance biopsy was associated with TAC-C_0 (odds ratio (OR): 0.69, 95% confidence interval (CI): 0.50–0.96; $p = 0.027$). In the second surveillance biopsy, inflammation was associated with time below the therapeutic range (OR: 1.05 and 95% CI: 1.01–1.10; $p = 0.023$). Interstitial inflammation in scarred areas (i-IFTA score ≥ 1) was not associated with surrogates of TAC exposure/metabolism. Progression of interstitial fibrosis/tubular atrophy (IF/TA) was observed in 35 cases (41.2%). Multivariate regression logistic analysis showed that mean C/D (OR: 0.48; 95% CI: 0.25–0.92; $p = 0.026$) and IF/TA in the first biopsy (OR: 0.43, 95% CI: 0.24–0.77, $p = 0.005$) were associated with IF/TA progression between biopsies. A low C/D ratio is associated with IF/TA progression, suggesting that TAC nephrotoxicity may contribute to fibrosis progression in well immunosuppressed patients. Our data support that TAC exposure is associated with inflammation in healthy kidney areas but not in scarred tissue.

Keywords: tacrolimus; renal transplantation; protocol biopsies; concentration dose ratio; time in therapeutic range; coefficient of variation

1. Introduction

Renal transplantation is the best treatment for end-stage renal disease, since it is associated with a better long-term patient survival and a higher quality of life at a lower cost than dialysis techniques [1]. Since the beginning of the present century and following

international guidelines (Kidney Disease: Improving Global Outcomes; KDIGO) maintenance immunosuppression in most renal transplant units is based on the combination of tacrolimus (TAC) and mycophenolate mofetil (MMF) either with or without low-dose steroids [2]. One important limitation for this strategy is that TAC is a drug with a narrow therapeutic window, and the optimal whole blood target levels (TAC-C_0) during follow up have not been properly defined. Low TAC exposure during the first year has been associated with a higher risk of clinical and subclinical acute rejection and a higher risk of the development of HLA donor-specific antibodies (DSA) in a prospective randomized clinical trial [3]. Additionally, registry studies have shown an association between low TAC exposure and poorer long-term graft survival [4]. Meanwhile, high TAC exposure has been associated with nephrotoxicity, viral infections, and cancer among other toxicities [5,6]. Additionally, tacrolimus is a drug with a low bioavailability (20–30%), and its metabolism rate is related with different genes [7].

To analyze the complex relationship between TAC exposure and clinical outcomes, different parameters can be employed. During the last years, a high intra-patient variability of tacrolimus trough levels during the first years after transplant has been associated with acute rejection, development of de novo DSA, chronic active antibody-mediated rejection, and poorer long-term graft survival [8,9]. It was sustained that patients with high intra-patient variability are outside the therapeutic range during longer periods of time [10,11], and this fact may be especially harmful for those kidneys with a higher HLA incompatibility [12].

In the other hand, to explore the association between tacrolimus metabolism and clinical outcomes, different pharmacokinetic and pharmacodynamic models have been developed, taking into consideration clinical variables such as hematocrit, serum albumin, age, gender, or body mass index and polymorphisms of the most relevant genes encoding cytochrome-P450 enzymes, CYP3A4 and CYP3A5 [13]. However, it has been shown that a simple measurement such as the concentration dose ratio of tacrolimus (C/D) can predict risk to develop tacrolimus side effects. Some studies support that fast metabolizers of tacrolimus are more prone to show nephrotoxicity and polyoma BK nephropathy [14,15].

Clinical monitoring of renal transplants relies mainly on serial determinations of serum creatinine and proteinuria, therapeutic drug monitoring of treatments with a narrow therapeutic window (e.g., TAC-C_0), determination of HLA antibodies by Luminex technology, and monitoring of the viral load of cytomegalovirus and polyoma BK virus. During the last decades, some centers have incorporated surveillance biopsies performed at different time points to detect the presence of subclinical inflammation and progression of renal scarring [16]. It has been proposed that in patients treated with tacrolimus and MMF, lower tacrolimus trough levels are associated with subclinical inflammation [17] and the progression of interstitial fibrosis and tubular atrophy (IF/TA) [18]. Similarly, a higher intra-patient variability of TAC-C_0 has also been associated with a faster progression of IF/TA [19,20]. Finally, it has been shown that high tacrolimus clearance is a risk factor for the development of IF/TA in a large cohort of patients monitored by surveillance biopsies [21].

In this study, we explore the association between histological lesions and different surrogates of tacrolimus exposure/metabolism in a cohort of low immunological risk renal transplants treated with prolonged-release TAC (PR-TAC), MMF, and steroids monitored by paired surveillance biopsies. To analyze TAC exposure, we employed TAC-C0 at the time of each biopsy and assessed the intra-patient variability of TAC trough levels and time in/above/below the therapeutic range during follow up. To analyze TAC metabolism, we employed the tacrolimus C/D.

2. Patients and Methods

2.1. Patients

We conducted a prospective, longitudinal, observational study in renal transplants performed at our Renal Transplant Unit since January 2012 until December 2018. All living

and donor deceased single kidney transplants performed in adult patients were considered. A first surveillance biopsy at 3–5 months after transplantation was performed in patients fulfilling the following criteria: (a) serum creatinine lower than 2 mg/dL; (b) stable renal function defined as a variability of serum creatinine lower than 15% between the determination at the time of biopsy and the previous one; (c) urinary protein creatinine ratio lower than 1 g/g; (d) non-use of oral anticoagulants; (e) non-technical difficulties to perform a renal biopsy (e.g., patients with large abdominal obesity, patients with large perirenal hematomas or patients with an idiomatic barrier were not considered) and (f) written informed consent. A second biopsy was performed in all patients at 12–18 months regardless of renal function and proteinuria.

This protocol was approved by the Ethics Committee of our center (PR/AG 104/2011), was performed in accordance with the Declaration of Helsinki, and is consistent with the Principles of the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

For the present study, we considered low immunological risk renal transplants, which are defined as the absence of donor-specific HLA antibodies at the time of transplant or having received a desensitization treatment before transplant in the case of living donors treated with prolonged-release tacrolimus (PR-TAC), mycophenolate mofetil (MMF) or enteric-coated mycophenolic acid (EC-MPA) and steroids during follow up. Patients treated with mTOR inhibitors (sirolimus or everolimus) from the day of transplant or switched to these drugs during follow up were also not considered.

2.2. Biopsies

Renal biopsies were performed under ultrasound guidance by trained radiologists with a 16-gauge automated needle. Three cores of tissue were obtained: one was processed for optical microscopy; one was embedded in OCT for immunofluorescence; and the other one was stored in RNA easy for molecular studies.

For optical microscopy, biopsies were embedded in formalin, paraffin-fixed, and 2–4 µm thick sections were stained with hematoxylin-eosin, periodic acid Schiff, Masson's trichrome and silver methenamine. Sample adequacy and histological lesions were evaluated according to the last update of the Banff criteria by the renal pathologists [22].

Interstitial inflammation in surveillance biopsies was defined as i-score $\geq$ 1, while biopsies without interstitial infiltrates (i-score = 0) were classified as no inflammation. IF/TA score (ci + ct) was calculated for each biopsy and progression of IF/TA between biopsies was defined as a difference in ci + ct score between the second and first surveillance biopsy $\geq$ 1. Arteriolar hyalinosis progression was defined as a difference of ah-score between the second and first surveillance biopsy $\geq$ 1.

Immunofluorescence studies were performed in 3-µm cryostat sections stained with FITC-conjugated anti-human IgG, IgA, IgM, C3, κ and λ light chain. C4d was stained with indirect immunofluorescence with a monoclonal antibody (Quidel, San Diego, CA, USA), and its deposition in peritubular capillaries was graded according to the Banff criteria. All biopsies were stained with an anti-SV40 antibody to discard BK polyomavirus nephropathy.

2.3. Immunosuppression

Standard immunosuppression included the use of induction therapy for all renal transplants. Recipients of a first renal transplant with a calculated panel reactivity antibodies (cPRA) <50% received 20 mg of Basiliximab (Simulect®; Novartis, Basel, Switzerland) at days 0 and 4. Patients with previous transplants and/or with positive non-DSA anti-HLA antibodies with a cPRA $\geq$ 50% and/or receiving grafts from a deceased donor after cardiac death were treated with three to five doses of rabbit anti-thymocyte globulin (Thymoglobulin®; Sanofi-Aventis, Paris, France) on alternate days to reach a total dose of 3–6 mg/kg.

For the present study, we considered patients receiving maintenance immunosuppression based on the combination of PR-TAC (Advagraf®; Astellas Pharma, Meppel, The Netherlands), MMF (Cellcept®; Roche Pharmaceuticals, Basel, Switzerland), or EC-

MPA (Myfortic®; Novartis, Basel, Switzerland), and steroids at the time of both surveillance biopsies. All patients received MMF 1 g bid (or EC-MPA 0.72 g bid) during the first month and 500 mg bid thereafter (or EC-MPA 0.36 g bid). In cases of suspected clinical intolerance to MMF or EC-MPA, further reductions of doses were done. The day of transplant patients received 250–500 mg of methylprednisolone, 125 mg at day 1 and 20 mg of prednisone at day 2. Thereafter, prednisone dose was progressively tapered to reach a daily dose of 0.1 mg/kg at 3 months and maintained during follow-up.

2.4. Therapeutic Drug Monitoring (TDM)

Tacrolimus trough levels in whole blood (TAC-C_0) were measured by CMIA immunoassay (Abbott Laboratories®; Abbott Park, IL, USA), and the intra-assay and inter-assay coefficient of variation was lower than 6%. Target TAC-C_0 were 8–12 ng/mL during the first 3 months after transplant and 6–10 ng/mL thereafter.

For the present study, TAC-C_0 monitoring done at the following time periods was considered: weekly during the first month, every two weeks during the second and third months, monthly from 4 to 6 months, and every 2 months from 6 to 12 months. At the time of biopsies additional samples were obtained. To analyze the relationship between tacrolimus exposure/metabolism and histological lesions, we analyzed the intra-patient variability of tacrolimus concentration, the time on therapeutic range, and the concentration dose ratio (Figure 1).

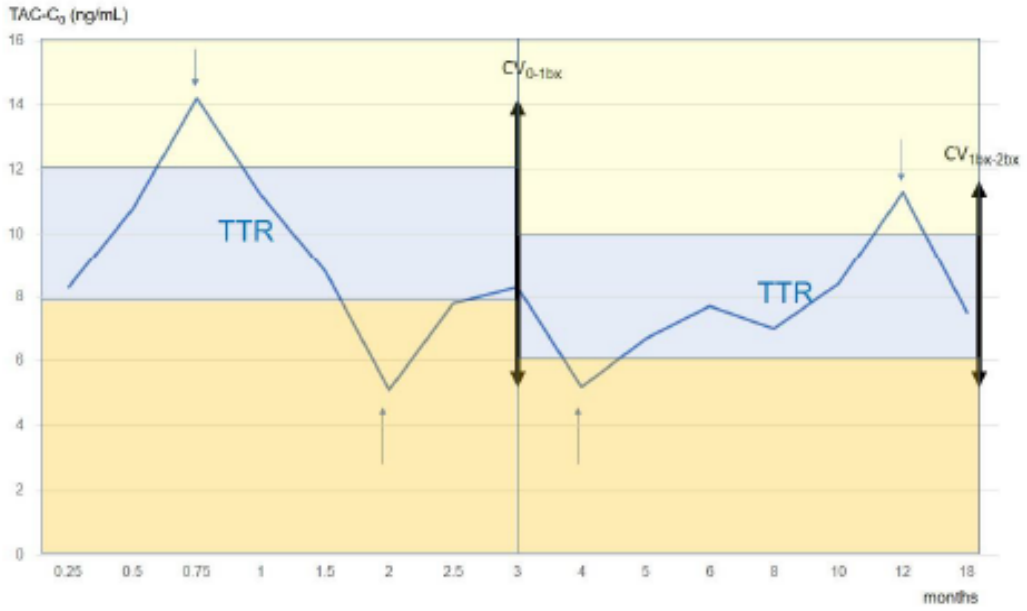

Figure 1. Evolution of tacrolimus trough levels during follow up in a patient from our cohort. TTR (blue area), time in the therapeutic range; times above (yellow area) and below (orange area) the therapeutic range are also shown. CV 0-1bx, coefficient of variability of tacrolimus between transplant and the first surveillance biopsy (29.3%); CV 1bx-2bx, coefficient of variability of tacrolimus between the first and the second surveillance biopsies (24.5%). In the *x*-axis, time of follow up (months) is presented, while in the *y*-axis, tacrolimus trough levels (ng/mL) are depicted. Arrows indicated determinations above and below the therapeutic range.

Coefficient of variability (CV). Intra-patient variability of tacrolimus trough levels was evaluated as the CV calculated according to: CV (%) = (SD/mean) * 100. For the present study, we considered CV of TAC-C_0 between the first week after transplant and the first protocol biopsy and between both biopsies. The mean number of TAC-C_0 determinations for the first period was 7.8 ± 1.7 (range 6–11) and for the second period, it was 6.1 ± 0.8 (range 5–7).

Time in therapeutic range (TTR). The linear interpolation method according to Rosendaal [23] was used to calculate TTR as well as the time below or above the therapeutic range. Briefly, this method assumes that a linear relationship exists between each measured value and then assigns a specific value for each day between tests (Figure 1). According to our immunosuppression schedule, the TAC-C_0 therapeutic range was defined as 8–12 ng/mL during the first three months and 6–10 ng/mL thereafter. We calculated for each patient the number of days in, above, and below the therapeutic range and expressed the result as the percentage time for each studied period (from the first week until the first biopsy and between both biopsies).

Tacrolimus concentration dose ratio (C/D). Tacrolimus dose was recorded at 3, 6, and 12 months as well as the day of biopsies. The mean tacrolimus concentration dose ratio (C/D; ng/mL/mg) was calculated as the mean of concentration dose ratio at each time point.

2.5. Clinical Variables

Demographic characteristics of donors and recipients as well as transplant-related variables were recorded. Anti-HLA antibodies at the time of transplant and at the time of each biopsy were determined by Luminex technology using the product Lifecodes LifeScreen Deluxe (Gen-Probe; San Diego, CA, USA), and IgG specificities were examined by single antigen beads testing with Lifecodes Luminex single antigen class I and class II kits. At the time of each biopsy, serum creatinine, TAC-C_0, and tacrolimus and MMF dose were recorded. In patients receiving EC-MPA, equimolar doses to MMF were used (720 mg of EC-MPA is equivalent to 1000 mg of MMF). Cytomegalovirus (CMV) infection was managed according to the international criteria [24]. Briefly, valganciclovir prophylaxis during the first 3–6 months was employed in high-risk patients (seropositive donor to seronegative recipient and patients treated with ATG) and a pre-emptive strategy with CMV viremia monitoring at each visit for the remaining patients. Monitoring of polyoma virus BK infection was done by the determination at each visit of BK viruria or BK viremia for those with increasing viral load in urine (>10^7 log). In patients with increasing BK viral load, reduction of MMF and tacrolimus dose and/or switch to low tacrolimus dose and mTOR inhibitors was done according to the attending physician.

2.6. Statistics

Variables were described as frequencies, median, and interquartile range or mean and standard deviation for categorical, non-normally distributed continuous variables and normally distributed continuous variables, respectively. To compare paired data (Fisher exact test, Wilcoxon T test, or paired *t*-test) and unpaired data (Fisher exact test, Mann–Whitney U test, and *t*-test) appropriate tests were employed. Logistic regression analysis was employed to analyze the associations between histological lesions and clinical and TDM data. For multivariate logistic regression analysis, those variables with a p-value < 0.20 in the univariate analysis were considered. All tests were two-tailed, and a p-value < 0.05 was considered significant. Statistical analysis was done with Stata 13.1 software package (Stata Corp LP, College Station, TX, USA).

3. Results

3.1. Patients

During the study period, 692 renal transplants were performed at our center. The flow chart of included patients in the present study is shown in Figure 2. Demographic data and transplant-related variables from the studied cohort are shown in Table 1. Clinical data at the time of both surveillance biopsies as well as TDM are shown in Table 2. Renal function remains stable between biopsies. According to our protocol, tacrolimus doses and TAC-C_0 were lower in the second period. The CV of TAC-C_0 was lower, and TTR was higher during the second period. The bioavailability of TAC was slightly higher (higher C/D ratio) during the second period, but this difference did not reach statistical significance.

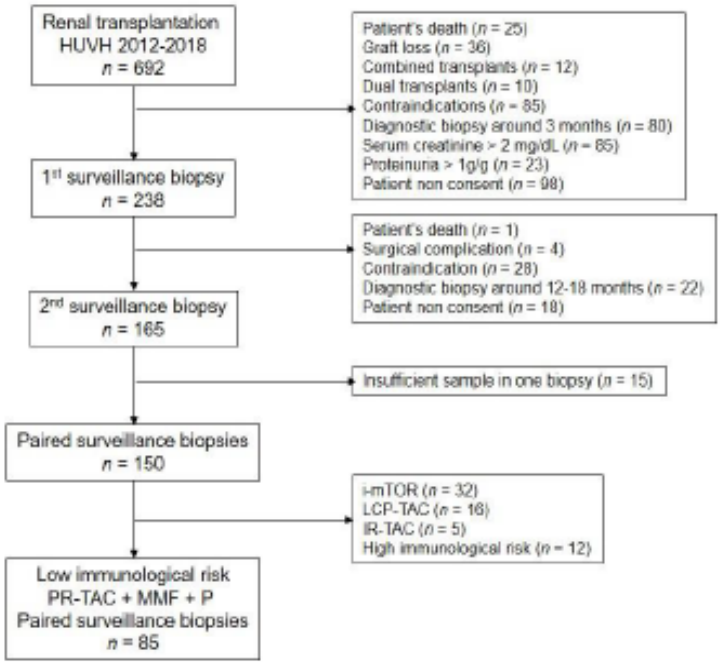

Figure 2. Flow chart of included patients. PR-TAC, prolonged-release tacrolimus; MMF, mycophenolate mofetil; P, prednisone; LCP-TAC, extended-release tacrolimus; IR-TAC, immediate-release tacrolimus; i-mTOR, inhibitors of mammalian target of rapamycin. Contraindications for the first surveillance biopsy include treatment with oral anticoagulants ($n = 33$), large abdominal obesity ($n = 26$), technical difficulties due to perirenal hematoma or lymphocele ($n = 9$), idiomatic barrier ($n = 13$), and horseshoe kidneys ($n = 4$). Indications for the use of mTOR inhibitors in this cohort was as follows: inclusion in a clinical trial containing i-mTOR de novo or early conversion ($n = 16$), polyoma BK viremia during follow up ($n = 6$), CMV viremia after prophylaxis in high-risk recipients ($n = 8$) and skin cancer ($n = 2$). High immunological risk patients were defined as those with HLA donor-specific antibodies at the time of transplant ($n = 7$) or receiving a desensitization treatment before transplant ($n = 5$).

Table 1. Donor and recipient characteristics as well as transplant related-variables from the studied cohort.

Variable	n = 85
Donor type (SCD/ECD/LD)	42 (49.4%)/30 (35.3%)/13 (15.3%)
Donor age (years)	52 ± 15
Donor gender (m/f)	50 (58.8%)/35 (41.2%)
Recipient age (years)	53 ± 13
Recipient gender (m/f)	66 (77.6%)/19 (22.4%)
First transplant/re-transplant	76 (89.4%)/9 (10.6%)
Primary renal disease (GN/ADPKD/diabetes/others/unknown)	21 (24.7%)/22 (25.9%)/8 (9.4%)/12 (14.1%)/22 (25.9%)
Class I HLA mismatch (A + B)	2.6 ± 0.9
Class II HLA mismatch (DR)	0.9 ± 0.7
Induction (Basiliximab/ATG)	52 (61.2%)/33 (38.8%)
DGF (no/yes)	80 (94%)/5 (6.0%)
T cell-mediated rejection (no/yes)	81 (95.3%)/4 (4.7%)
CMV infection (no/viremia/disease)	71 (83.5%)/10 (11.8%)/4 (4.7%)
Polyoma BK virus infection (no/viremia/nephropathy)	79 (92.9%)/6 (7.1%)/0 (0%)
Post-transplant diabetes mellitus (no/yes)	61 (79.2%)/16 (20.8%)

SCD, standard criteria deceased donor; ECD, expanded criteria deceased donor; LD, living donor; GN, glomerulonephritis; ADPKD, autosomal dominant polycystic kidney disease; DGF, delayed graft function; CMV, cytomegalovirus.

Table 2. Clinical data at the time of the first and second surveillance biopsies of the studied cohort ($n = 85$).

Variable	First Biopsy	Second Biopsy	*p*-Value
Time of biopsy (months)	4.2 ± 1.9	17.3 ± 3.6	n.a.
Serum creatinine (mg/dL)	1.31 ± 0.32	1.27 ± 0.28	0.106
eGFR (mL/min/1.73 sqm)	61.8 ± 17.7	63.4 ± 17.1	0.220
Urine P/C ratio (mg/g)	260 ± 170	320 ± 370	0.057
Tacrolimus dose (mg/day)	6.6 ± 3.9	4.9 ± 3.1	0.001
MMF dose (mg/kg/day)	13.4 ± 3.4	12.9 ± 3.3	0.096
TAC-C0 (ng/mL)	9.6 ± 2.4	8.5 ± 2.3	0.002
CV of TAC-C0 (%)	31 ± 13	20 ± 14	0.001
Time in TR(%)	55 ± 24	70 ± 25	0.001
Time above TR (%)	35 ± 25	26 ± 29	0.066
Time below TR (%)	10 ± 13	4 ± 11	0.004
C/D (ng/mL/mg)	2.00 ± 1.42	2.19 ± 1.02	0.119
De novo DSA (%)	0	0	n.a.

eGFR, estimated glomerular filtration rate by MDRD-4 formula; urine P/C ratio, urine protein creatinine ratio; TAC-C0, tacrolimus trough levels; CV of TAC-C0, coefficient of variability of tacrolimus trough levels from the first week until the first biopsy and between the first and the second biopsies; TR, percentage of time in/above/below the therapeutic range until the first biopsy and between the first and the second biopsies; C/D, concentration dose ratio of tacrolimus; DSA, donor specific antibodies.

There exists a correlation between the different TDM methods employed in this study (correlation matrix at the time of both biopsies are shown in Tables S1 and S2).

3.2. Biopsies

The prevalence of subclinical rejection was low in both surveillance biopsies. In the first biopsy, there was one single case of T cell-mediated rejection (TCMR) grade IIA (isolated v-lesion in a patient without tubule-interstitial inflammation or microvascular inflammation and without HLA antibodies). There were no cases reaching criteria for tubulo-interstitial TCMR ($i \geq 2$ and $t \geq 2$). Borderline changes suspicious of TCMR ($i \geq$ and $t \geq 1$ but lower than i2t2) were observed in eight cases (9.4%) in the first biopsy and in nine cases in the second one (10.6%). Isolated inflammation ($i \geq 1$ and $t = 0$) was observed in 11 (12.9%) and in five cases (5.9%) in the first and second biopsies. Isolated tubulitis ($i = 0$ and $t \geq 1$) was observed in six (7.1%) and in 13 (15.3%) cases in the first and second biopsy, respectively. No tubule-interstitial inflammation ($i = 0$ and $t = 0$) was observed in 60 (70.6%) and 58 (68.2%) in the first and second biopsies.

In the first biopsy, there were 43 cases (50.6%) with IF/TA ($ci \geq 1$ and $ct \geq 1$) that was mild ($n = 38$), moderate ($n = 4$), and severe ($n = 1$). In the second biopsy there were 57 cases (67.1%) with mild ($n = 41$), moderate ($n = 13$), and severe ($n = 3$) IF/TA. Criteria for chronic TCMR were fulfilled in two cases (2.3%) in the first and in five cases (5.9%) in the second biopsy. The degree of inflammatory lesions in any renal compartment did not change between biopsies, although tubulitis tended to be higher in the second biopsy. By contrast, chronic lesions including IF/TA and arteriolar hyalinosis significantly increased between biopsies (Table 3).

3.3. Interstitial Inflammation and TDM

Patients displaying interstitial inflammation (i-score ≥ 1) in the first surveillance biopsy had lower TAC-C0 levels and higher CV-TAC until biopsy. There was no association between TTR or time above/below the therapeutic range and tubule-interstitial inflammation (Table 4).

By logistic regression analysis, the presence of inflammation in the first surveillance biopsy was associated with TAC-C_0 levels (odds ratio (OR): 0.69 and 95% confidence interval (CI): 0.50–0.96; $p = 0.027$) while CV-TAC until biopsy was not included. In the second surveillance biopsy, the time below the therapeutic range and TAC-C_0 were lower in patients with inflammation (Table 5).

Table 3. Banff scores in the first and second surveillance biopsies (n = 85).

Variable	First Biopsy	Second Biopsy	p-Value
Glomerular sections (n)	13 ± 8	13 ± 8	0.881
Global glomerulosclerosis (%)	7 ± 10	8 ± 11	0.541
Glomerulitis (g)	0.12 ± 0.36	0.15 ± 0.45	0.580
Interstitial infiltrate (i)	0.27 ± 0.54	0.17 ± 0.37	0.118
Tubulitis (t)	0.18 ± 0.42	0.31 ± 0.58	0.086
Endothelialitis (v)	0.01 ± 0.11	0	0.320
Peritubular capillaritis (ptc)	0.13 ± 0.37	0.18 ± 0.47	0.413
Arteriolar hyalinosis (ah)	0.38 ± 0.64	0.65 ± 0.79	0.008
Transplant glomerulopathy (cg)	0	0.04 ± 0.24	0.183
Interstitial fibrosis (ci)	0.73 ± 0.77	1.00 ± 0.84	0.002
Tubular atrophy (ct)	0.76 ± 0.59	1.08 ± 0.73	0.001
Vascular intimal thickening (cv)	0.62 ± 0.91	0.67 ± 0.85	0.636
Mesangial matrix increase (mm)	0.02 ± 0.15	0.01 ± 0.11	0.567
i-IFTA	1.28 ± 1.17	1.39 ± 1.21	0.494
t-IFTA	0.36 ± 0.55	0.51 ± 0.67	0.128

i-IFTA. Interstitial inflammation in areas of interstitial fibrosis and tubular atrophy; t-IFTA, tubulitis in areas of interstitial fibrosis and tubular atrophy.

Table 4. Clinical and therapeutic drug monitoring data in patients with (i-score ≥1) and without (i-score = 0) interstitial inflammation in the first surveillance biopsy.

Variable	No Inflammation (n = 66)	Inflammation (n = 19)	p-Value
Time of biopsy (months)	4.3 ± 1.5	3.7 ± 2.9	0.167
Serum creatinine (mg/dL)	1.34 ± 0.33	1.20 ± 0.26	0.106
eGFR (mL/ min/ 1.73 sqm)	61 ± 17	65 ± 19	0.369
Urine P/C ratio (mg/g)	270 ± 180	220 ± 130	0.252
Tacrolimus dose (mg/day)	6.7 ± 3.9	5.4 ± 3.5	0.184
MMF dose (mg/kg/day)	13.2 ± 3.3	13.5 ± 3.8	0.795
TAC-C0 (ng/mL)	10.0 ± 2.4	8.3 ± 2.2	0.007
CV of TAC-C0 (%)	29 ± 12	37 ± 15	0.030
Time in TR (%)	56 ± 26	55 ± 21	0.889
Time above TR (%)	36 ± 26	33 ± 21	0.642
Time below TR (%)	8 ± 13	12 ± 14	0.246
C/D (ng/mL/mg)	1.88 ± 1.18	2.45 ± 2.03	0.124

eGFR, estimated glomerular filtration rate by MDRD-4 formula; urine P/C ratio, urine protein creatinine ratio; TAC-C0, tacrolimus trough levels; CV of TAC-C0, coefficient of variability of tacrolimus trough levels from the first week until the first biopsy; TR, percentage of time in/above/below the therapeutic range from the first week until the first biopsy; C/D, concentration dose ratio of tacrolimus.

By logistic regression analysis, the presence of inflammation in the second surveillance biopsy was associated with the time below the therapeutic range (OR: 1.05 and 95% CI: 1.01–1.10; p = 0.023), while TAC-C_0 was not included. Interstitial inflammation in scarred areas (i-IFTA score) was not associated with surrogates of TAC exposure/metabolism (Tables S3 and S4).

3.4. IF/TA Progression and TDM

Progression of IF/TA was observed in 35 cases (41.2%). Univariate analysis showed that IF/TA progression was associated with the mean C/D ratio and IF/TA score in the first biopsy (Tables 6 and 7). Multivariate logistic regression analysis showed that mean C/D (OR: 0.48; 95% CI: 0.25–0.92; p = 0.026) and IF/TA in the first biopsy (OR: 0.43, 95% CI: 0.24–0.77, p = 0.005) were associated with IF/TA progression.

Table 5. Clinical and therapeutic drug monitoring data in patients with (i-score ≥ 1) and without (i-score = 0) interstitial inflammation in the second surveillance biopsy.

Variable	No Inflammation (*n* = 71)	Inflammation (*n* = 14)	*p*-Value
Time of biopsy (months)	17.4 ± 3.8	16.4 ± 2.5	0.380
Serum creatinine (mg/dL)	1.26 ± 0.26	1.33 ± 0.37	0.405
eGFR (mL/min/1.73 sqm)	64.4 ± 17.4	58.2 ± 15.2	0.217
Urine P/C ratio (mg/g)	301 ± 348	423 ± 494	0.270
Tacrolimus dose (mg/day)	5.0 ± 3.3	4.3 ± 2.1	0.426
MMF dose (mg/kg/day)	12.7 ± 3.5	13.1 ± 1.7	0.691
TAC-C0 (ng/mL)	8.7 ± 2.3	7.4 ± 1.7	0.059
CV of TAC-C0 (%)	20 ± 15	19 ± 7	0.826
Time in TR (%)	70 ± 27	74 ± 19	0.534
Time above TR (%)	27 ± 27	14 ± 21	0.076
Time below TR (%)	3 ± 10	12 ± 15	0.005
C/D (ng/mL/mg)	2.22 ± 1.04	2.04 ± 0.82	0.557

eGFR, estimated glomerular filtration rate by MDRD-4 formula; urine P/C ratio, urine protein creatinine ratio; TAC-C0, tacrolimus trough levels; CV of TAC-C0, coefficient of variability of tacrolimus trough levels from the first until the second biopsy; TR, percentage of time in/above/below the therapeutic range from the first until the second biopsy; C/D, concentration dose ratio of tacrolimus.

Table 6. Clinical and therapeutic drug monitoring data in patients with and without interstitial fibrosis and tubular atrophy progression between biopsies.

Variable	No Progression (*n* = 50)	IFTA Progression (*n* = 35)	*p*-Value
Donor age (years)	54 ± 16	50 ± 13	0.285
Donor type (DD/LD)	43 (86%)/7 (14%)	29 (82.8%)/6 (17.2%)	0.382
Recipient age (years)	54 ± 13	50 ± 14	0.184
1st transplant/re-transplant	45 (90%)/5 (10%)	31 (88.6%)/4 (11.4%)	0.551
HLA-DR mismatch	1.0 ± 0.7	0.9 ± 0.7	0.856
Cold ischemia time (hours)	15 ± 7	14 ± 8	0.850
Induction (IL2-RA/ATG)	29 (58%)/21 (42%)	22 (62.9%)/13 (37.1%)	0.558
DGF (no/yes)	48 (96%)/2 (4%)	32 (91.4%)/3 (8.6%)	0.399
T-cell mediated rejection (no/yes)	47 (94%)/3 (6%)	34 (97%)/1 (3%)	0.640
Time of biopsy (months)	17.7 ± 4.1	16.6 ± 2.7	0.156
Serum creatinine (mg/dL)	1.25 ± 0.29	1.29 ± 0.27	0.582
eGFR (mL/min/1.73 sqm)	64 ± 18	63 ± 16	0.881
Urine P/C ratio (mg/g)	310 ± 340	350 ± 425	0.622
Tacrolimus dose 1st bx (mg/day)	5.8 ± 3.8	7.4 ± 3.8	0.058
Tacrolimus dose 2nd bx (mg/day)	4.3 ± 2.9	5.8 ± 3.2	0.023
MMF dose (mg/kg/day)	12.9 ± 3.2	12.7 ± 3.4	0.836
TAC-C0 1st biopsy (ng/mL)	9.8 ± 2.7	9.4 ± 1.9	0.515
TAC-C0 2nd biopsy (ng/mL)	8.1 ± 1.7	9.1 ± 2.8	0.053
CV of TAC-C0 until first biopsy (%)	32 ± 13	30 ± 13	0.503
CV of TAC-C_0 between biopsies (%)	21 ± 18	18 ± 7	0.305
Time in TR between biopsies (%)	73 ± 25	67 ± 27	0.353
Time above TR between biopsies (%)	22 ± 25	29 ± 29	0.318
Time below TR between biopsies (%)	5 ± 12	4 ± 9	0.798
Mean C/D (ng/mL/mg)	2.3 ± 1.3	1.7 ± 0.7	0.019

DD, deceased donor; LD, living donor; DGF, delayed graft function; eGFR, estimated glomerular filtration rate by MDRD-4 formula; urine P/C ratio, urine protein creatinine ratio; TAC-C0, tacrolimus trough levels; CV of TAC-C0, coefficient of variability of tacrolimus trough levels; TR, percentage of time in/above/below the therapeutic range; C/D, concentration dose ratio of tacrolimus.

Table 7. Banff scores in the first and second surveillance biopsies in patients with and without IF/TA progression between biopsies.

Variable	No Progression ($n = 50$)	IFTA Progression ($n = 35$)	p-Value
g+ptc score 1st biopsy	0.32 ± 0.74	0.14 ± 0.43	0.207
i+t score 1st biopsy	0.56 ± 0.91	0.29 ± 0.62	0.125
i-IFTA score 1st biopsy	1.43 ± 1.19	1.03 ± 1.12	0.124
ci+ct score 1st biopsy	1.90 ± 1.27	0.89 ± 0.93	0.001
ah-score 1st biopsy	0.40 ± 0.61	0.34 ± 0.69	0.686
g+ptc score 2nd biopsy	0.24± 0.56	0.46 ± 0.95	0.188
i+t score 2nd biopsy	0.48 ± 0.89	0.46 ± 0.70	0.899
ci+ct score 2nd biopsy	1.60 ± 1.28	2.71 ± 1.64	0.001
i-IFTA score 2nd biopsy	1.20 ± 1.27	1.69 ± 1.08	0.073
ah-score 2nd biopsy	0.69 ± 0.77	0.60 ± 0.85	0.598

g, glomerulitis; ptc, peritubular capillaritis; i, interstitial inflammation in non-scarred cortex; t, tubulitis; ci, interstitial fibrosis; ct, tubular atrophy; i-total, interstitial inflammation in the whole cortex; i-IFTA, interstitial inflammation in scarred cortex; ah, arteriolar hyalinosis.

3.5. Arteriolar Hyalinosis Progression and TDM

During follow-up, arteriolar hyalinosis progressed in 31 cases (36.5%). Clinical and TDM data were not different among patients with and without arteriolar hyalinosis progression.

4. Discussion

A relationship between progression of IF/TA, evaluated by means of sequential surveillance biopsies, and surrogates of TAC exposure or metabolism, such as low TAC trough levels [18], high TAC variability [19,20], and C/D ratio [21], have been described in separate studies. In the present study, all these parameters were evaluated in the same cohort of patients to further characterize their relative contribution to IF/TA progression. We did not observe any association between TAC trough levels, TAC variability or time below the therapeutic range, and progression of IF/TA; while low C/D ratio, a surrogate of fast metabolizers, was associated with IF/TA progression. To interpret these observations, it should be taken into consideration that in the present cohort, TAC trough levels were relatively high in comparison to other immunosuppressive schedules [25], and time below the therapeutic range was lower than in previous reported studies. Regarding the relationship between high TAC variability and IF/TA, this relationship has only been observed in patients with a high variability and long-time periods of TAC levels below the therapeutic range [10]. Thus, the lack of a relationship between surrogates of TAC exposure and progression of IF/TA in the present study is consistent with the above-mentioned studies.

Subclinical inflammation has been associated with IF/TA progression [26,27], an increased risk for clinical rejection [28], the appearance of de novo donor-specific antibodies, especially in patients with a high number of HLA mismatches [12,29], the development of chronic antibody-mediated rejection [30], and decreased graft survival [31]. However, in this study, we failed to show an association between interstitial inflammation and IF/TA progression. This result may be explained by the low incidence and severity of inflammation. Only in one out of 170 biopsies did we observe subclinical rejection in a patient with isolated v-lesion, which is a histological phenotype that does not represent true rejection in a significant proportion of cases [32,33]. Approximately, there were 10% of early and late biopsies that displayed borderline changes and an additional 13% and 6% of early and late biopsies showed mild inflammation without tubulitis. Accordingly, in approximately 80% of cases, there was no interstitial inflammation. The relatively low incidence and severity of subclinical inflammation may be ascribed to the relatively high TAC trough levels that remained most of the time within the therapeutic range and to the low immunological risk of these set of patients [3,17,34]. We arbitrarily employed an i-score ≥ 1 as the threshold to distinguish between inflammation and no inflammation. This deci-

sion was based on previous observations showing that an i-score ≥ 1 is associated with decreased graft survival, while isolated tubulitis has a minor and controversial influence on outcome [35–37]. Despite the low degree of inflammation, TAC levels were significantly higher in early biopsies without inflammation, and the time below the therapeutic range was longer in late biopsies, suggesting that even in low immunological risk patients receiving a high TAC schedule, interstitial inflammation in healthy interstitial areas is modulated by TAC exposure. This observation constitutes an argument to sustain that subclinical inflammation in healthy areas may represent the balance between alloimmune response and immunosuppression. On the contrary, we did not observe any association between inflammation in scarred areas and TAC exposure or metabolism. The relationship between immunosuppressive treatment and inflammation in scarred tissue (i-IFTA) has been evaluated in few studies. In the study conducted by Lefaucheur et al. [38], the withdrawal of steroids, MMF, or calcineurin inhibitors at 6 months was associated with a higher risk of i-IFTA at 1 year. Similarly, in the study conducted by Nankivell et al. [39], tacrolimus was associated with a lower risk of i-IFTA than cyclosporine. In our study, all patients were treated with tacrolimus, MMF, and steroids until the second biopsy. Thus, in patients receiving a power immunosuppression, there was no association between i-IFTA and tacrolimus exposure, suggesting that other non-controlled factors in the present study contribute to the development of this lesion. Interstitial fibrosis, with independence of its trigger, is frequently associated with mononuclear cell infiltration [40–42]. In studies comparing gene expression in for-cause renal allograft biopsies with inflammation in scarred and unscarred areas, it was observed that inflammation in unscarred areas correlated with transcripts associated with cytotoxic T cells, while inflammation in scarred areas correlated with B cell, plasma cell, mast cell, and injury-repair transcripts [43,44]. This difference may be explained by the selective effect of tacrolimus on activated T cells [45,46]. Our data raise the question of whether the presence of inflammation in healthy kidney areas may facilitated TAC treatment adjustment to patient's needs, following a personalized medicine approach. In the other hand, these data argue against the utility of inflammation in scarred areas to adjust TAC dose.

In the present study, there was an association between low C/D ratio, a surrogate of TAC metabolism, and the progression of IF/TA between both biopsies. Patients with a low C/D ratio represent fast metabolizers, since they need a high dose to reach the therapeutic levels. It has been previously described that a low C/D ratio is associated with poorer allograft function and a higher incidence of BK nephropathy and decreased allograft survival [15,47–49]. The pharmacokinetic curve in fast metabolizers is characterized by a higher peak TAC concentration (Cmax) in comparison with low metabolizers to reach a similar TAC-C0 [50,51]. Thus, it has been proposed that the progression of IF/TA in these patients can reflect TAC nephrotoxicity. In fact, according to our data, tacrolimus metabolism and not tacrolimus exposure contributes to IF/TA progression. The progression of IF/TA was also associated with the severity of IF/TA in the first biopsy, but this is an expected result that depends on the definition of ci and ct-scores according to the Banff criteria. Scoring for ci and ct is done according to the extension of interstitial fibrosis and tubular atrophy in the available tissue cortex as: ≤5% (ci = 0 and ct = 0); 6–25% (ci = 1 and ct = 1); 26–50% (ci = 2 and ct = 2); and >50% (ci = 3 and ct = 3). This kind of classification implies that patients without IF/TA in the first biopsy (ci and ct ≤ 5%) will have a higher risk of progression than patients with mild IF/TA (ci and ct of 6–25%) considering that the range of this last category is wider than the former. This association between IF/TA in the first biopsy and the risk of progression in biopsies performed later has been described in previous studies with paired surveillance biopsies [52].

Our study has some limitations. Preimplantation biopsies were not available, and we were not able to characterize IFTA progression from the donor to the first surveillance biopsy. Additionally, a 24-h pharmacokinetic study was not done to evaluate whether fast metabolizers (lower C/D ratio) have a higher tacrolimus Cmax than poor metabolizers (higher C/D ratio).

5. Conclusions

In conclusion, low C/D ratio is associated with IF/TA progression but not TAC trough levels, TAC variability, or time below the therapeutic range. Thus, TAC nephrotoxicity may contribute to fibrosis progression in well immunosuppressed patients. Additionally, we confirm that high TAC levels decrease inflammation in healthy kidney areas but not in scarred areas, pointing out that inflammation in scarred areas is not responsive to TAC exposure.

Supplementary Materials: The supplementary tables are available online at https://www.mdpi.com/2077-0383/10/1/141/s1. Table S1: Correlation matrix of surrogate variables of tacrolimus exposure/metabolism at the time of the first biopsy. Table S2: Correlation matrix of surrogate variables of tacrolimus exposure/metabolism at the time of the second biopsy. Table S3: Inflammation and tubulitis in areas of interstitial fibrosis/tubular atrophy in the first surveillance biopsy and surrogate variables of tacrolimus exposure/metabolism. Table S4: Inflammation and tubulitis in areas of interstitial fibrosis/tubular atrophy in the second surveillance biopsy and surrogate variables of tacrolimus exposure/metabolism.

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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 Hospital Universitari Vall d'Hebron (protocol code PR-AG 104/2011; 29/07/2011)

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.

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Artículo 3

Betty Chamoun, Irina B. Torres, Alejandra Gabaldón, Thomas Jouvé, Maria Meneghini, José M. Zúñiga, Joana Sellarés, Manel Perelló, Daniel Serón, Oriol Bestard, Francesc Moreso.

4.3 Expression of rejection-associated transcripts in early protocol renal transplant biopsies are associated with tacrolimus exposure and graft outcome.

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Expression of Rejection-Associated Transcripts in Early Protocol Renal Transplant Biopsies Is Associated with Tacrolimus Exposure and Graft Outcome

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Abstract: Subclinical inflammation in protocol biopsies relates to tacrolimus exposure and human leukocyte antigen (HLA) matching. We aimed to characterize transcripts associated with rejection and tacrolimus exposure and the latter's association with transplant outcomes. We tested whether gene expression is associated with rejection using strictly normal protocol biopsies (n = 17) and biopsies with T cell-mediated rejection (TCMR) or antibody-mediated rejection (ABMR) according to Banff criteria (n = 12). Subsequently, we analyzed these transcripts in a set of 4-month protocol biopsies (n = 137) to assess their association with donor and recipient characteristics, the intensity of immunosuppression, and the graft outcome. Differential expression (false discovery rate (FDR) < 0.01, fold change (FC) > 3) between normal and rejection biopsies yielded a set of 111 genes. In the protocol biopsy cohort (n = 137), 19 out of these 111 genes correlated with tacrolimus trough levels at the time of biopsy (TAC-C_0), and unsupervised analysis split this cohort into two clusters. The two clusters differed in donor age and tacrolimus trough levels. Subclinical rejection, including borderline lesions, tended to occur in the same cluster. Logistic regression analysis indicated that TAC-C_0 at the time of biopsy (OR: 0.83, 95%CI:0.72–0.06, $p = 0.0117$) was associated with cluster 2. In a follow-up averaging 70 ± 30 months, this patient group displayed a significant decline in renal function ($p = 0.0135$). The expression of rejection-associated transcripts in early protocol biopsies is associated with tacrolimus exposure and a faster decline in renal function.

Keywords: renal transplantation; biopsies; rejection; gene expression; tacrolimus

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1. Introduction

Routine graft monitoring in renal transplantation relies on non-invasive biomarkers such as serum creatinine, proteinuria, and HLA antibodies. More than 30 years ago, to explore the feasibility of histological monitoring, some centers started programs of protocol biopsies and observed that there were grafts with stable function depicting histological changes of rejection, leading to the definition of subclinical rejection (SCR) [1–3]. During the cyclosporine era, SCR was prevalent (>30%) and it was proven that its treatment better preserves renal function [2,4]. However, a clinical trial addressing SCR treatment in patients on modern immunosuppression with tacrolimus/MMF yielded limited clinical

benefit due to the low rate of SCR [5]. For this reason, it has also been explored whether minor histological changes are associated with graft outcome. In a study including a large set of 6-month protocol biopsies (n = 957), it was shown that interstitial inflammation (i-score > 0) in otherwise normal protocol biopsies is associated with a significantly lower 15 y graft survival, comparable to SCR or interstitial fibrosis/tubular atrophy (IF/TA) with inflammation [6].

Although the evaluation of renal biopsies based on the Banff classification for renal transplant pathology has been refined since 1991 [7], some uncertainties persist, notably concerning the presence of borderline changes suspicious for T cell-mediated rejection (TCMR) and the incomplete phenotypes of antibody-mediated rejection (ABMR). To further characterize underlying mechanisms leading to different histological phenotypes, an analysis of the transcriptome has been incorporated [8–11]. It has been shown that molecular diagnostics allow the detection of transcript sets strongly associated with TCMR and have proven useful in differentiating borderline infiltrates likely to lead to the development of overt TCMR and/or graft fibrosis [12]. Recently, we have shown that a rejection-associated gene expression score is present in 83% of protocol biopsies with SCR but only in 17% of protocol biopsies with borderline changes [13]. Importantly, to distill information from RNA microarrays that evaluate thousands of genes, the Banff group has delineated gene sets related to TCMR, ABMR, tissue-repair injury, and other pathways implicated in graft dysfunction [9–14].

In studies of serial protocol biopsies performed in renal transplants, the prevalence of SCR is maximal during the initial three months, progressively decreases until the first year, and persists in a small number of patients after the first year. Risk factors associated with SCR are the number of human leukocyte antigen mismatches, the degree of sensitization, retransplantation, the presence of previous clinical acute rejection episodes, and the immunosuppressive regimen being lower in patients treated with tacrolimus and MMF [3]. Our group and others have shown that reduced exposure to tacrolimus and/or MMF is associated with a higher incidence of subclinical inflammation in protocol biopsies performed during the first year [15,16]. Furthermore, while certain studies have linked SCR to HLA ABDR allelic mismatch [17], disparities at the molecular level might offer more informative insights [18]. Notably, in liver transplant recipients, reduced immunosuppression exposure and an increased number of HLA epitope mismatches between donor and recipient have been implicated in the molecular pathogenesis of subclinical liver allograft damage driven by an interferon gamma-orchestrated cellular immune response [19].

In this current study, we employ microfluidic cards to scrutinize the transcriptome of a predefined set of genes related to different histological diagnoses (mainly TCMR and ABMR) previously described by the Banff group [14]. We aim to determine whether transcripts increase or decrease in biopsies with rejection, reflecting changes in resident and/or infiltrating cells. To achieve this, we compare gene expression normal-protocol biopsies and for-cause biopsies that meet the Banff criteria for TCMR or ABMR. Subsequently, we quest these transcripts in a large set of early protocol biopsies to evaluate whether gene expression is associated with donor and recipient characteristics, including the intensity of immunosuppression and donor–recipient HLA mismatch at the allelic or molecular level.

2. Results

2.1. Patients and Biopsies

In the present study, we have included three groups of patients: patients with a strictly normal early protocol biopsy (group I, n = 17); patients with a biopsy for cause displaying rejection, either TCMR or ABMR, (group II, n = 12); and a large cohort of patients with a protocol biopsy displaying different histological phenotypes (group III, n = 137). Donor and recipient characteristics as well as transplant-related variables from the three studied groups are shown in Table 1. Biopsies were evaluated according to the 2019 Banff criteria [9]. The timing of the biopsy and laboratory data at the time of the biopsy are detailed in Table 2. In the rejection group II (n = 12), there was a mix of cases with TCMR (n = 5), active ABMR

(n = 5), and mixed rejection (n = 2). In group III, which contained only protocol biopsies (n = 137), the histological Banff categories were as follows: non-specific changes (n = 40), subclinical TCMR (n = 5), subclinical ABMR (n = 3), borderline changes (n = 16), interstitial fibrosis and tubular atrophy (IF/TA) without interstitial inflammation (n = 59) and IF/TA with interstitial inflammation (IF/TA + i) (n = 14). These results agreed with the prevalence of the different histological phenotypes in the cohort of early protocol biopsies (n = 397) obtained at our center (40.1%, 3.1%, 2.7%, 11.5%, 34.3%, and 8.3%, respectively).

Table 1. Donor and recipient characteristics as well as transplant-related variables in the 3 study groups.

Variable	Group I (n = 17)	Group II (n = 12)	Group III (n = 137)
Donor type (BDD/DCD/LD)	10/4/3	7/2/3	85/34/18
Donor age (years)	45 ± 18	59 ± 15	57 ± 14
Donor gender (m/f)	10/7	4/8	77/60
Recipient age (years)	46 ± 13	50 ± 16	55 ± 14
Recipient gender (m/f)	10/7	6/6	91/46
First transplant/retransplant	15/2	7/5	117/20
Primary renal disease (GN/ADPKD/diabetes/others/unknown)	3/5/0/4/5	4/1/1/2/4	28/18/11/25/55
Class I HLA mismatch (A + B)	2.5 ± 0.9	2.1 ± 0.9	2.8 ± 1.0
Class II HLA mismatch (DR)	1.1 ± 0.5	1.3 ± 0.6	1.1 ± 0.6
Induction (basiliximab/thymoglobulin)	8/9	4/8	77/60
Cold ischemia time	14.3 ± 6.7	13.0 ± 7.0	13.4 ± 6.8
Delayed graft function (no/yes)	16/1	10/2	123/19
Previous episodes of rejection (no/yes)	17/0	9/3	134/7
DSA at the time of transplant (no/yes)	15/2	11/1	130/7
CMV infection (no/viremia/disease)	14/2/1	9/3/0	115/18/4

Group I—normal protocol biopsies; Group II—biopsies with rejection; group III—protocol biopsies with different histological phenotypes BDD—brain death donor; DCD—donation after circulatory death; LD—living donor; GN—glomerulonephritis; ADPKD—autosomal dominant polycystic kidney disease; DSA—HLA donor-specific antibodies; CMV—cytomegalovirus. Mean ± SD or raw numbers are employed to describe variables.

Table 2. Data at the time of biopsy in the 3 study groups.

Variable	Group I (n = 17)	Group II (n = 12)	Group III (n = 137)
Time of biopsy (months)	4.7 ± 1.7	43 ± 55	4.4 ± 1.4
Serum creatinine (mg/dL)	1.22 ± 0.31	2.72 ± 1.95	1.44 ± 0.32
eGFR (mL/min/1.73 sqm)	66.6 ± 23.0	35.1 ± 21.1	52.2 ± 14.6
Urine P/C ratio (mg/g)	260 ± 190	1890 ± 1340	265 ± 192
DSA at the time of biopsy (no/yes)	17/0	8/4	133/4
Tacrolimus dose (mg/day)	6.8 ± 4.2	7.6 ± 5.9	6.5 ± 4.2
TAC-C_0 (ng/mL)	8.8 ± 2.0	7.9 ± 3.6	9.3 ± 2.7
MMF dose (g/day)	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.2

Group I—normal protocol biopsies; Group II—biopsies with rejection; Group III—protocol biopsies with different histological phenotypes; eGFR—estimated glomerular filtration rate according to the CKD-EPI formula; urine P/C ratio; protein to creatinine ratio in a spot morning urine sample; DSA—HLA donor-specific antibodies; TAC-C_0—tacrolimus trough levels; MMF—mycophenolate mofetil. Mean ± SD or raw numbers are employed to describe variables.

To evaluate whether there was an association between subclinical inflammation and donor/recipient characteristics, transplant-related variables, or immunosuppression, we compare patients in whom the protocol biopsy showed interstitial inflammation (i > 0) and patients in whom the protocol biopsy did not show interstitial infiltrates (i = 0). Among the evaluated variables, subclinical inflammation was associated with prolonged cold ischemia time ($p = 0.040$) and lower tacrolimus trough levels (TAC-C_0) at the time of biopsy ($p = 0.002$) (Tables 3 and 4).

Table 3. Donor/recipient characteristics and transplant-related variables according to interstitial inflammation in the protocol biopsy.

Variable	i-Score = 0 (n = 99)	i-Score ≥1 (n = 38)	*p*-Value
Donor type (BDD/DCD/LD)	58/27/14	27/7/4	0.490
Donor age (years)	57 ± 14	56 ± 15	0.837
Donor gender (m/f)	56/43	21/17	0.793
Recipient age (years)	55 ± 14	57 ± 14	0.453
Recipient gender (m/f)	65/34	26/12	0.759
First transplant/retransplant	85/14	32/6	0.807
Class I HLA mismatch (A + B)	2.8 ± 0.9	2.9 ± 1.0	0.521
Class II HLA mismatch (DR)	1.1 ± 0.6	1.2 ± 0.6	0.233
Class I Eplet mismatch	14 ± 6	14 ± 8	0.845
Class II Eplet mismatch	15 ± 10	17 ± 15	0.305
PIRCHE-II class I	49 ± 27	49 ± 29	0.914
PIRCHE-II class II	34 ± 25	34 ± 22	0.996
DSA at the time of transplant (no/yes)	96/3	34/4	0.074
Induction (Basiliximab/ATG)	53/46	24/14	0.367
Cold ischemia time	12.6 ± 6.9	15.3 ± 6.2	0.040
DGF (no/yes)	87/12	31/7	0.339
TCMR before protocol biopsy (no/yes)	94/5	36/2	0.960

BDD—brain death donor; DCD—donation after circulatory death; LD—living donor; DGF—delayed graft function; DSA—donor-specific antibodies. Mean ± SD or raw numbers are employed to describe variables.

Table 4. Data at the time of biopsy according to interstitial inflammation in the protocol biopsy.

Variable	i-Score = 0 (n = 99)	i-Score ≥1 (n = 38)	*p*-Value
Time of biopsy (months)	4.3 ± 1.4	4.6 ± 1.7	0.169
Serum creatinine (mg/dL)	1.5 ± 0.3	1.4 ± 0.3	0.747
eGFR (mL/min/1.73 m^2)	52 ± 14	53 ± 16	0.790
Urine P/C ratio (mg/g)	275 ± 206	239 ± 148	0.331
DSA at the time of biopsy (no/yes)	96/3	37/1	0.901
Tacrolimus dose (mg/day)	6.7 ± 4.5	6.0 ± 3.3	0.425
TAC-C_0 (ng/mL)	9.7 ± 2.7	8.2 ± 2.2	0.002
C/D tacrolimus (ng/mL/mg)	1.72 (1.06–2.80)	1.54 (1.05–1.98)	0.220
CV TAC-C_0 from day 7 to biopsy (%)	36.5 ± 23.3	36.6 ± 14.3	0.990
Time in TR (%)	67 ± 31	69 ± 33	0.738
Time above TR (%)	24 ± 28	17 ± 26	0.184
Time below TR (%)	7 ± 14	10 ± 18	0.303
MMF dose (g/day)	0.9 ± 0.2	0.9 ± 0.2	0.735
eGFR decline (mL/min/1.73 m^2/year)	−0.8 ± 4.3	−1.1 ± 2.9	0.163

eGFR—estimated glomerular filtration rate by MDRD-4 formula; urine P/C ratio—urine protein creatinine ratio; TAC-C_0—tacrolimus trough levels at the time of biopsy; CV of TAC-C_0—coefficient of variability of tacrolimus; C/D—concentration dose ratio of tacrolimus. Mean ± SD or raw numbers are employed to describe variables.

Donor and recipient demographics, HLA mismatches at the allelic or molecular level, the presence of delayed graft function, the timing of the biopsy, and renal function did not differ between groups (Table 3). At the time of biopsy, three out of four patients with DSA displayed subclinical ABMR. Multivariate logistic regression analysis showed that TAC-C_0 (odds ratio [OR]: 0.76; 95% confidence interval [CI]: 0.63–0.92; p-value = 0.004) was associated with i-score > 0 while cold ischemia time was on the verge of significance (OR: 1.06; 95% CI:.0.99–1.13; p-value = 0.077).

2.2. Transcriptome Analysis by Microfluidics

The gene expression in the three groups of biopsies was firstly analyzed by principal component analysis (PCA), and it can be observed that biopsies from group I (normal) and biopsies from group II (TCMR/ABMR) cluster in different areas of the plot while the largest sample of protocol biopsies (group III) clusters in between (Figure 1). The most relevant genes in PCA were ADAMDEC1, CCL5, CLEC4C, CXCL13, and CXCL9 for component 1; and COL1A1, NPHS1, NPHS2, SLC22A2, and SLC4A1 for component 2. As expected, the gene expression comparison between group I and group II (adjusted p-value < 0.01 and fold change > 3) yielded as many as 111 differentially expressed genes (Supplementary Table S1). These 111 genes extracted from the list provided by the Banff group [14] were mainly related to TCMR (72 genes), ABMR (16 genes), and tissue damage (8 genes).

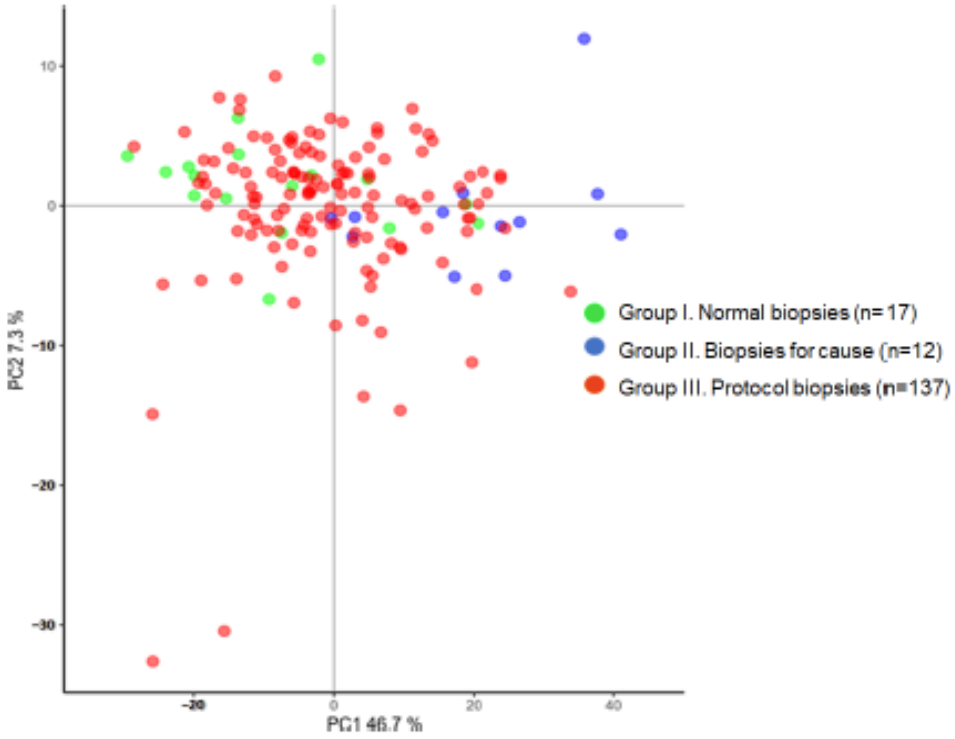

Figure 1. Principal component analysis with the expression of 307 genes in the different study groups.

2.3. Transcriptome Analysis and Clinical Variables

We evaluated whether there was an association between TAC-C_0 at the time of biopsy and the 111 genes associated with rejection (Supplementary Table S2). In group I (strictly normal protocol biopsies) there was a close correlation between TAC-C_0 at the time of biopsy and expression of IKZF3 and CD2 genes (Figure 2). Conversely, in group II (biopsies for cause with TCMR/ABMR) there was no correlation between TAC-C_0 at the time of biopsy and the expression of any gene. Finally, 19 genes mainly related to TCMR (12 out of 19) correlated with TAC-C_0 in group III.

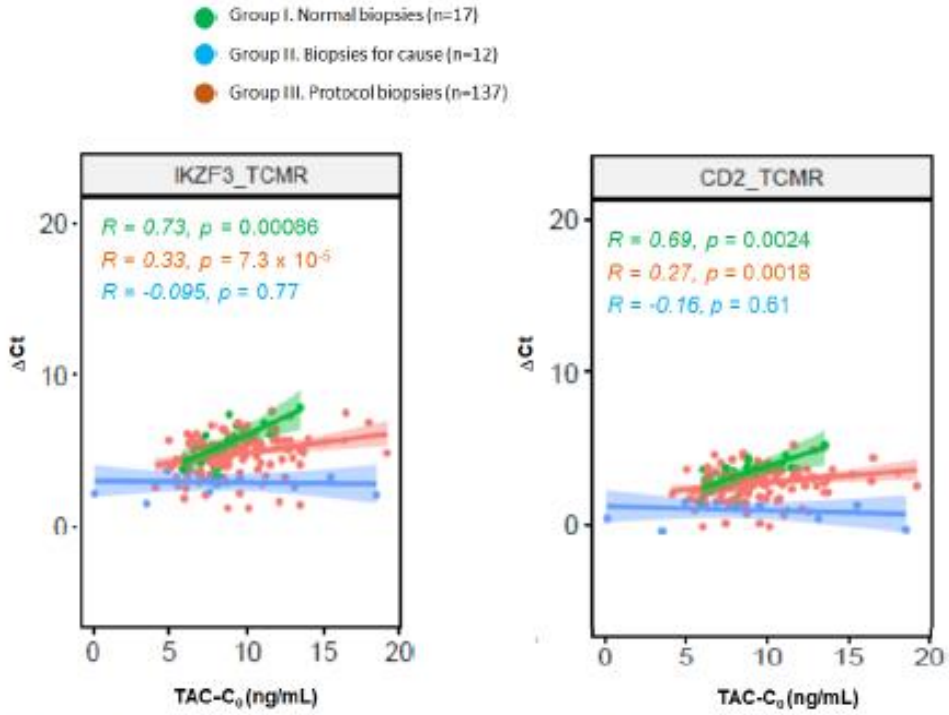

Figure 2. Correlation between tacrolimus trough levels (TAC-C_0) at the time of biopsy and the expression of IKZF3 and CD2 genes in the different study groups.

Unsupervised cluster analysis allowed two clusters of biopsies to be defined, one containing all normal protocol biopsies (with one exception) and the other containing all biopsies with rejection (Figure 3).

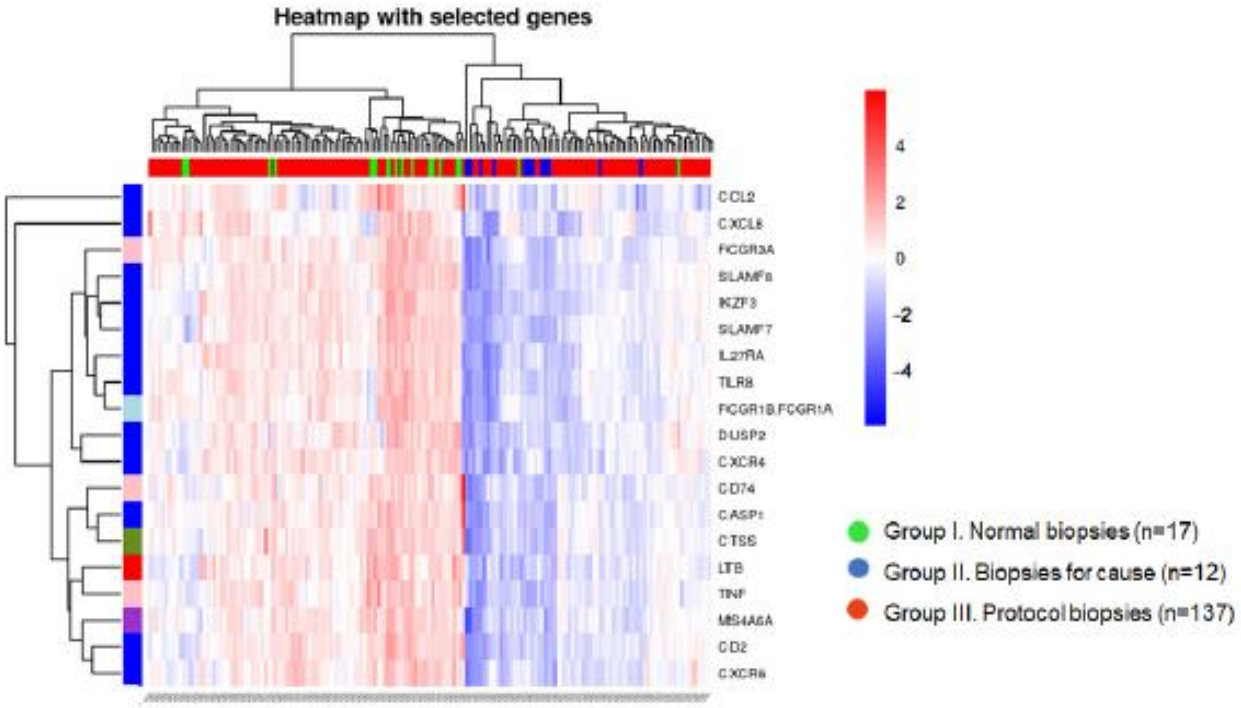

Figure 3. Heatmap of the 19 genes correlated with tacrolimus trough levels (TAC-C_0) at the time of biopsy in the different study groups.

Protocol biopsies from Group III were distributed in a similar proportion in both clusters (77 in Cluster 1 and 60 in Cluster 2). We compared both clusters of biopsies from group III and observed that older donors and lower tacrolimus trough levels at the time of biopsy were grouped in cluster 2 (Table 5).

Table 5. Donor/recipient characteristics, transplant-related variables, and data at the time of biopsy in patients from both clusters according to gene expression.

Variable	Cluster 1 (n = 77)	Cluster 2 (n = 60)	*p*-Value
Donor type (BDD/DACD/LD)	47/17/13	38/17/5	ns
Donor age (y)	54 ± 13	60 ± 15	0.0316
Patient age (y)	54 ± 13	57 ± 15	ns
Patient sex (m/f)	53/24	38/22	ns
First transplant/retransplant	68/9	49/11	ns
Class I HLA mismatch (A + B)	2.7 ± 1.0	3.0 ± 1.0	0.039
Class II HLA mismatch (DR)	1.1 ± 0.7	1.2 ± 0.6	0.571
HLA eplet class I mismatch	13 ± 6	15 ± 7	0.061
HLA eplet class II mismatch	14 ± 11	16 ± 9	0.335
HLA AbV eplet DRB mismatch	2.8 ± 2.4	3.6 ±2.4	0.059
HLA AbV eplet DQB mismatch	2.6 ± 2.6	2.5 ± 2.3	0.757
PIRCHE-II class I	48 ± 28	52 ± 28	0.446
PIRCHE-II class II	34 ±28	34 ± 19	0.999
Induction (basiliximab/thymoglobulin)	44/33	33/27	ns
DGF (n/y)	67/10	51/9	ns
TCMR before protocol biopsy (n/y)	72/5	68/2	ns
eGFR (mL/min/1.73 sqm) biopsy	53 ± 13	51 ± 16	ns
Urinary protein/creatinine (g/g) biopsy	0.24 ± 0.17	0.30 ± 0.24	ns
TAC-C_0 (ng/mL) biopsy	9.8 ± 2.6	8.6 ± 2.6	0.0133
CV of TAC-C_0 from day 7 to biopsy (%)	34.9 ± 22.4	39.2 ± 20.5	0.2483
Time in TR (%)	68 ± 32	50 ± 30	0.430
Time above TR (%)	25 ± 30	15 ± 25	0.304
Time below TR (%)	5 ± 15	12 ± 18	0.070
MMF dose (g/day)	0.9 ± 0.2	0.9 ± 0.2	ns
eGFR decline (mL/min/1.72 m^2/year)	−0.2 ± 3.7	−1.9 ± 4.1	0.0145

BDD—brain death donor; DCD—donation after circulatory death; LD—living donor; DGF—delayed graft function; TCMR T cell-mediated rejection; N—normal; BL—borderline lesions; SCR—subclinical rejection; IFTA—interstitial fibrosis—and tubular atrophy; eGFR—estimated glomerular filtration rate; TAC-C_0—tacrolimus trough levels at the time of biopsy. Mean ± SD or raw numbers are employed to describe variables.

Noticeably, subclinical rejection including borderline lesions (19 out of 24 cases) and biopsies with IF/TA + i (8 out of 14 cases) also tended to be grouped in cluster 2 (Figure 4). Logistic regression analysis showed that only TAC-C_0 at the time of biopsy (OR: 0.83, 95% CI: 0.72–0.96, *p*-value = 0.0117) was associated with cluster 2.

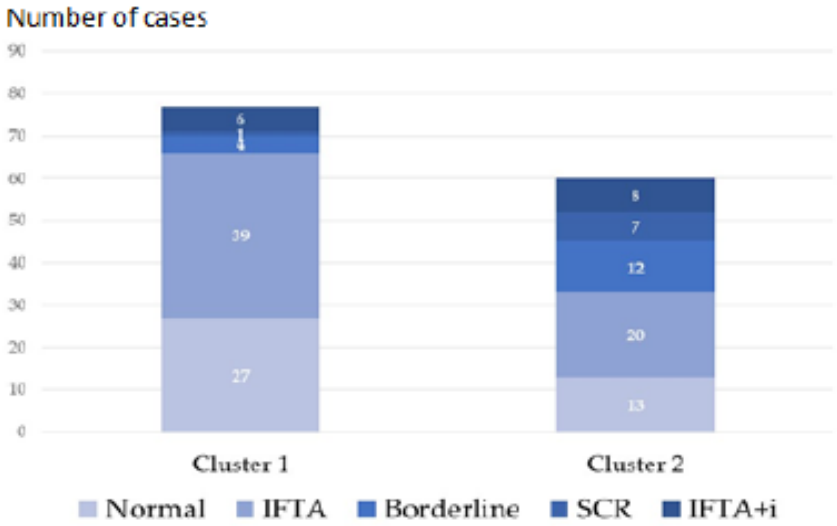

Figure 4. Histological diagnosis in the two clusters of biopsies was defined according to the gene expression of the 19 genes correlated with tacrolimus trough levels (TAC-C_0) at the time of biopsy.

2.4. Renal Outcome and Protocol Biopsies

At a mean follow-up of 70 ± 30 months, the mean decline in renal function was −0.9 ± 3.9 mL/min/1.73 m^2/year in the group of patients with a protocol biopsy (group III). While the mean annual decline in renal function did not differ significantly between patients with (i > 0) or without inflammation (−1.1 ± 2.9 vs. −0.8 ± 4.3 mL/min/1.73 m^2/year, Table 4), it was significantly higher in patients from cluster 2 than in patients from cluster 1 (−1.9 ± 4.1 vs. −0.2 ± 3.7 mL/min/1.73 m^2/year; $p = 0.0135$; Table 5). Moreover, this difference was independent of the presence of inflammation in the protocol biopsy (cluster 1 with i = 0 (n = 66) −0.20 ± 3.9; cluster 1 with i > 0 (n = 11) +0.19 ± 1.99; $p = 0.691$; and cluster 2 with i = 0 (n = 33) −2.03 ± 4.8; cluster 2 with i > 0 (n = 27) −1.71 ± 3.23; $p = 0.763$).

The total number of patients developing de novo DSA during follow-up in our cohort was low (n = 4; 2.9%). Kaplan–Meier analysis showed that patients with and without inflammation have a non-different rate of development of de novo DSA ($p = 0.876$), while this rate tended to be higher in patients from cluster 2 ($p = 0.073$).

3. Discussion

We conducted a prospective study on a set of 4-month protocol biopsies to evaluate whether validated rejection-associated transcripts are associated with tacrolimus exposure at the time of biopsy. The main findings of our study are that we confirm the discrimination capacity between normal and rejection biopsies of a large set of these genes and that the expression of 19 rejection-associated transcripts in early protocol biopsies is associated with tacrolimus exposure at the time of biopsy. Cluster analysis using this set of 19 genes identified a pool of patients with a higher proportion of inflammatory phenotypes, including TCMR, borderline lesions, and IFTA with inflammation. Interestingly, patients from this cluster had less exposure to tacrolimus and displayed a faster decline in renal function during follow-up. The low rate of de novo DSA development in our cohort (2.9%) limits further analysis of its association with subclinical inflammation. Thus, our results suggest that adjusted immunosuppression during the early months after transplantation favors a better control of the inflammatory response without deleterious effects on renal function in the mid-term.

Tacrolimus is the mainstay of immunosuppressive regimens for kidney transplantation since it prevents T cell activation and proliferation. Although tacrolimus reduces the acute rejection rate and improves short-term outcomes after kidney transplantation, it is associated with both acute and chronic nephrotoxicity and triggers serious side effects. Although monitoring of tacrolimus exposure relies on clinical practice for determining trough levels,

there is no agreement on the target levels during the first year in renal transplant recipients. While the largest clinical trial supported the minimization of tacrolimus exposure [20], one randomized clinical trial has shown that in case of steroid discontinuation and MMF reduction, maintaining TAC-C_0 > 7 ng/mL after the fourth month reduces the risk of acute rejection and appearance of de novo DSAs without increasing renal toxicity [15]. Similarly, in low-immunological-risk renal transplants treated with TAC, reduced MMF, and low-dose steroids, TAC-C_0 levels are associated with subclinical inflammation in patients monitored by protocol biopsies [16]. Additionally, it has been described that the effect of tacrolimus trough levels was modulated by the recipient's baseline alloimmune risk, as defined by their class II HLA donor–recipient eplet mismatch [18].

In the present study, we analyzed whether interstitial inflammation is associated with clinical characteristics of donors and recipients as well as with transplant-related variables. In our cohort, the presence of interstitial inflammation was associated with lower TAC-C_0 at the time of biopsy and with longer cold ischemia time, but it was not associated with mid-term renal function deterioration or the development of de novo DSA. Importantly, in our cohort, as in several others [6,16], few cases met the criteria for subclinical borderline rejection (11.7%) or TCMR/ABMR (5.8%). Since the presence of interstitial inflammation (i > 0) in otherwise normal biopsies has been associated with 15-year death-censored graft survival [6] in a similar way to SCR, we chose this threshold for our analysis. Notably, in other studies including patients treated with a steroid-free regimen, the incidences of borderline rejection and TCMR were significantly more frequent (31% and 20.8%) [21]. In this study, the authors did not find associations between TAC-C_0 and subclinical inflammation, but it should be noted that at the time of the 3-month protocol biopsy, the TAC-C_0 average was close to 10 ng/mL [22]. In this study, SCR within the first post-transplant year is associated with a significantly greater hazard of subsequent clinical rejection and death-censored graft loss. On the contrary, other studies have shown that T cell-mediated inflammation detected in protocol biopsies mostly reflects the injury–repair response to implantation stresses and has little relationship with future events and outcomes [23]. Acute kidney injury (AKI) after renal transplantation can also induce interstitial infiltration and tubulitis [24] leading to a histological picture indistinguishable from that of TCMR. In this sense, in our cohort of protocol biopsies, we observed an association between interstitial inflammation and longer cold ischemia time. Thus, the presence of interstitial inflammation is uncommon in our cohort of low-immunological-risk kidney transplants maintaining steroids (27.7%) and it is associated with tacrolimus exposure and cold ischemia time, suggesting that both immune and non-immune factors may contribute to subclinical inflammation in well-functioning grafts.

The disagreement between different studies on the prevalence of subclinical inflammation and its association with later clinical outcomes is partly explained by the inclusion of different populations and different maintenance immunosuppression regimens. However, there is general agreement that conventional biopsy assessment is limited due to poor interobserver reproducibility of individual lesions [11,25]. To overcome these limitations, it has been proposed that molecular phenotyping be incorporated. The application of microarrays to transplant biopsies has been an ongoing effort by many groups and the interpretation of molecular changes aided by the understanding of their biological mechanisms led to the grouping of different transcripts [10,14,26,27]. To summarize information derived from RNA microarrays, which evaluate thousands of genes, in the last reports of the Banff meetings gene sets containing a few hundred genes related to TCMR, ABMR, tissue-repair injury and other pathways leading to graft dysfunction were described [6,26,28,29]. In the present study, we evaluated the panel of genes described in the Banff meeting in 2017 via RT-PCR [14]. As expected, we confirm the discrimination capacity of a high number of these genes (111 out of 308 evaluated genes) to differentiate normal protocol biopsies from biopsies for cause with rejection. In the principal component analysis, we observed that TCMR-selective genes expressed in activated effector T cells (ADAMDEC1) and genes encoding different cytokines and their receptors mainly related to TCMR (CCL5, CXCL13

and CXLC9) were the most relevant in component 1, while genes encoding matrix proteins and solute transporters (COL1A1, SCL22A2 and SCL4A1) were the most relevant in component 2.

Regarding the derived gene set, we were interested in evaluating its relationship with tacrolimus exposure at the time of biopsy. We found that 19 of these 111 genes, mainly related to TCMR, were mildly correlated with TAC-C_0, suggesting that a higher tacrolimus exposure contributes to a better control of subclinical inflammation. Interestingly, in the small set of normal protocol biopsies, we observed a close correlation between TAC-C_0 and the expression of 2 out of these 19 genes (IKZF3 and CD2, Figure 4) IKZF3, expressed mostly in the lymph and spleen, is found in several immune cell types, including B cells, NK cells, CD4+, and CD8+ T cells. It is expressed most strongly in B cells and studies of IKZF3 knockout mice indicate a critical role for IKZF3 in B-cell differentiation, maturation, proliferation, and T cell-dependent B-cell responses. IKZF3 is upregulated in pre-B cells, and it has been found to play a role in executing the transition from large pre-B cells to small pre-B cells during normal B-cell development. IKZF3 has also been found to play an important role in T cell regulation. It is expressed in interleukin-17-producing helper T cells and promotes differentiation through silencing of interleukin 2 production. Recently, it has been shown that IKZF3 is upregulated not only in ABMR but also in TCMR urinary cell specimens suggesting that B cells may play a more active role in TCMR than previously recognized, perhaps functioning as classical antigen-presenting cells [30–32]. The CD2 family of costimulatory and adhesion molecules has also been shown to play a significant role in the execution of an alloimmune response since it is constitutively expressed by all T cells and upregulated upon antigen recognition. Importantly, CD2 is more highly expressed on effector memory T cells relative to central memory T cells and therefore more effectively targets those cells that are poised to rapidly exert effector function upon encounter with cognate antigen. In addition to its role in facilitating the adhesion of T cells to antigen-presenting cells during the immunological synapse, CD2 ligation results in the direct transmission of co-stimulatory signals to promote T cell activation and differentiation [33,34]. Importantly, in the evaluated set of biopsies, the expression of these 19 genes associated with TAC-C_0 and split our protocol biopsy group into two clusters one containing all but one normal protocol biopsies and the other containing all rejection biopsies. The large set of protocol biopsies was distributed in a similar proportion in both clusters. Patients with protocol biopsies grouped in cluster 2 received a lower exposure to tacrolimus, showed more frequently an inflammatory phenotype, and displayed a faster decline of renal function in the mid-term. Thus, our results suggest that more adjusted immunosuppression during the early months after transplantation favors a better control of the inflammatory response and better preserving renal function in the mid-term.

Our effort to detect associations between gene expression, tacrolimus exposure, and HLA compatibility at the allelic or molecular level did not show significant associations in the multivariate analysis. It should be remarked that HLA typing in this cohort was performed according to clinical practice and thus, high-resolution HLA typing was not performed and the availability of HLA typing for all loci (especially DQ) was limited. However, in this cohort of successfully immunosuppressed renal transplant recipients, the number of patients developing de novo DSA was very low (2.9%) and although patients from cluster 2 tended to develop de novo DSA more frequently, this association did not reach statistical significance. Additionally, the present study has other important limitations, since associations between tacrolimus exposure and histological findings or gene transcripts were based on a single determination of TAC-C_0 on the day of biopsy and a more refined evaluation of tacrolimus pharmacokinetics (e.g., area under the time–concentration curve) or pharmacodynamics (e.g., calcineurin activity) was not done. Finally, patients in group II (acute rejection) underwent biopsies later in comparison to patients in the other groups (protocol biopsies), and the impact of biopsy timing on gene expression has been widely acknowledged [11,35,36].

4. Materials and Methods

4.1. Patients

We considered renal transplants included in a prospective, observational study with an early (at 3–5 months) protocol biopsy performed between 2012 and 2019 as previously described [16]. Surveillance biopsies were performed in patients fulfilling the following criteria: (a) serum creatinine lower than 2 mg/dL; (b) stable renal function defined as a variability of serum creatinine lower than 15% between the determination at the time of biopsy and the previous one; (c) urinary protein creatinine ratio lower than 1 g/g; (d) non-use of oral anticoagulants; (e) non-technical difficulties to perform a renal biopsy (e.g., patients with large abdominal obesity, patients with large perirenal hematomas or patients with an idiomatic barrier were not considered) and (f) written informed consent. Two control groups of biopsies were selected from our biobank: strictly normal protocol biopsies (group I, n = 17) and biopsies for cause with either TCMR or ABMR (group II, n = 12) to generate a set of genes associated with rejection. Later, we selected a large sample of protocol biopsies (group III, n = 142) to evaluate whether gene expression was associated with donor and recipient characteristics or the intensity of immunosuppression. The flowchart of the study is shown in Figure 5.

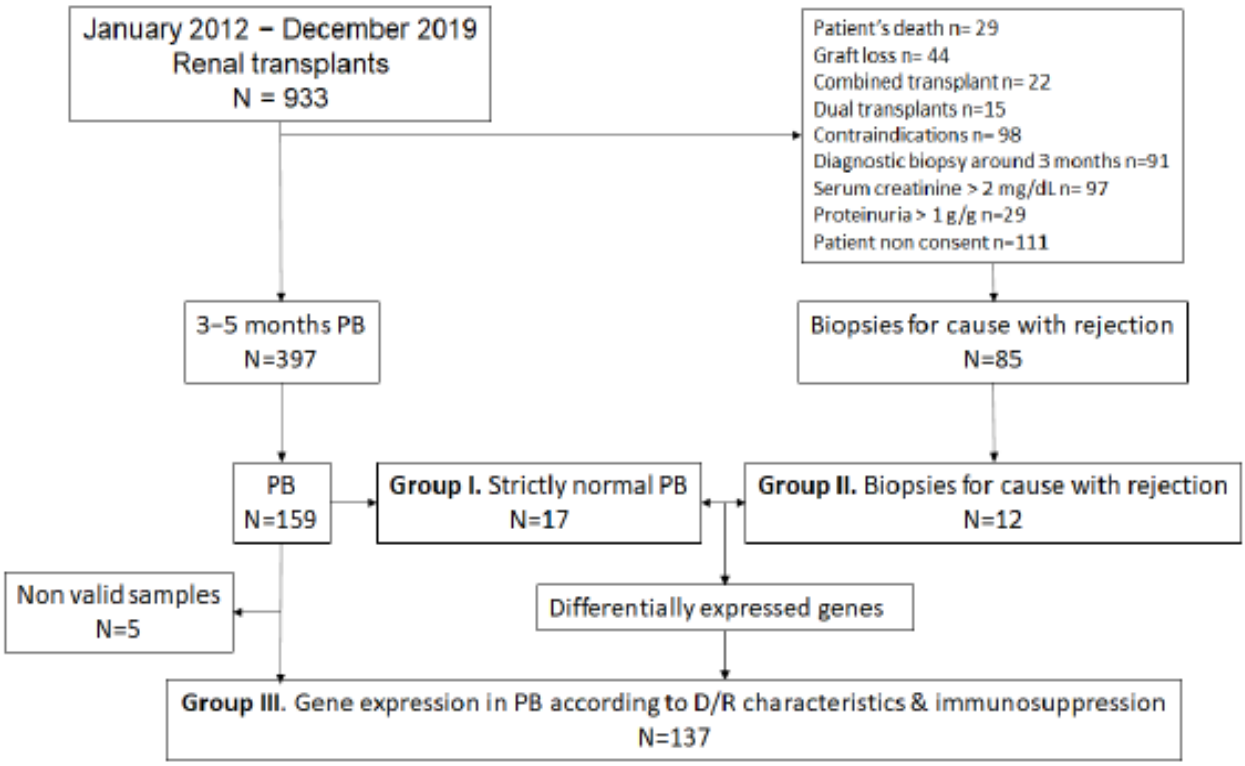

Figure 5. Flow chart of the patients and biopsies induded in the study. PB—protocol biopsies.

Demographic characteristics of donors and recipients as well as transplant-related variables were recorded. Patients were followed in the outpatient area and the decline in renal function was estimated from the linear regression of all available measurements and expressed as mL/min/1.73 m^2/year to adjust for the different timings of follow-up.

The present study has been approved by our Ethics Committee (Comité Etico de Investigación Clínica del Hospital Universitari Vall d'Hebron PR(AG)369/2014, approval date 1 December 2014) and all participants signed written informed consent. The study was conducted by the Declaration of Helsinki and adhered to the Principles of the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

4.2. HLA Typing and HLA Antibodies

The recipients' and donors' HLA typing was performed by DNA-based low-resolution typing with sequence-specific primers (SSP). For class I (A and B loci) and for class II (DR loci), results were available for all donor/recipient pairs. HLA C typing was available in 116 cases from donors and 47 cases for recipients. HLA DQ loci typing was available for 40 donors and 26 recipients.

The HLA Matchmaker program (Rene Duquesnoy, 2016, University of Pittsburgh Medical Center, Pittsburgh, PA HLA-ABC Eplet Matching Version 3.1 and DRDQDP Eplet Matching Program V3.1 from http://www.epitopes.net/downloads.html, (accessed on 17 November 2022) was used to calculate eplet scores. Donor and recipient typing was converted to high resolution using a local frequency table typed by sequence-based typing. Total numbers of incompatible eplets and antibody-verified eplets were calculated.

PIRCHE-II scores were calculated using version 3.3 from https://www.pirche.org (accessed on 22 May 2023). The NetMHCIIpan 3.0 algorithm predicted nonameric-binding cores of donor mismatched HLA-derived peptides that could bind to recipient HLA-DRB1. For cases with only low-resolution HLA typing, PIRCHE-II generates a potential high-resolution HLA typing and PIRCHE-II was calculated for each potential typing for both donors and recipients. These values were weighted by haplotype frequencies in the general population as validated in a previous study [37].

Anti-HLA antibodies on the day of transplant, biopsy, and during follow-up were determined using a single-antigen class-I and class-II flow beads-assay kit (LIFECODES, division of Immucor, Stanford, CA, USA). Beads with a normalized MFI > 500 were considered positive if (MFI/MFI lowest bead) > 5.

4.3. Immunosuppression

Induction and maintenance of immunosuppression with tacrolimus, MMF, and steroids were performed as previously described [16]. Target TAC-C_0 levels during the first 3 months were 8–12 ng/mL and 6–10 ng/mL thereafter. Exposure to tacrolimus was evaluated using a concentration dose ratio (C/D), coefficient of variation of TAC-C_0 until the day of biopsy, and TAC-C_0 at the time and time in/above/below therapeutic range of biopsy, as previously described [38].

4.4. Biopsies

Ultrasound-guided renal biopsies were performed with a 16G automated needle, and 3 cores of tissue were obtained: one was processed for optical microscopy; one was embedded in OCT for immunofluorescence and the other one was stored in RNA later. Histological lesions were evaluated according to the last Banff criteria [9] and the definition of borderline changes were foci of tubulitis (t1–t3) with mild interstitial inflammation (i1) or mild tubulitis (t1) with moderate–severe interstitial inflammation (i2–i3). C4d was stained with indirect immunofluorescence with a monoclonal antibody (Quidel, San Diego, CA, USA) and deposition in peritubular capillaries was graded according to the Banff criteria. The third core was stored with Ambion® RNAlater® Tissue Collection at −80 °C (Applied Biosystems, Austin, TX, USA).

4.5. Analysis Using Fluidigm Microfluidics Dynamic Arrays

Total RNA extraction, assessment of RNA quality, and cDNA synthesis were done as previously described [13]. The aim of the study was the quantitative analysis of 318 genes (308 target genes and 10 housekeeping genes: ECD, EIF1, FUBP3, GGNBP2, GNB1, RPN1, RPN2, SERBP1, UBC, UBE2D3) in the biopsies. The 308 target genes were selected from a list of identified, non-repeated prime gene lists reported in the Banff 2017 meeting [14]. For this purpose, we used the Biomark HD Nanofluidic Quantitative PCR (qPCR) system (Fluidigm Corporation, San Francisco, CA, USA) combined with GE 96.96 Dynamic Arrays IRCs. For sequence detection, predesigned Primetime qPCR primer assays or custom primer assays were used for amplification and detection using the EvaGreen fluorochrome.

The assays have been divided into four IDT assay plates and all housekeeping genes have been included in all plates. Samples were treated with Exonuclease I (Exo I) (Thermo Scientific EN0582, Willmington, DE, USA) to remove unincorporated primers. QIAGEN® Multiplex PCR Kit Cat N.206143 (Hilden, Germany) was used for the specific target amplification. According to the manufacturer's instructions, 13 genes were eliminated due to potential amplification of genomic DNA and 12 genes were also not considered due to lack of expression in more than half of the samples. The analysis of the expression of the cDNA was performed with Biomark HD Nanofluidic qPCR system (Fluidigm Corporation, San Francisco, CA, USA) combined with 96.96 Dynamic Arrays IFCs by employing the Master Mix Sso FastTM Eva Green® Supermix with Low ROX (Bio-Rad Laboratories, Hercules, CA, USA). The Ct (Cycle Threshold) data and the Quality Call of the amplification curve were determined by the Fluidigm Real-Time PCR Analysis Software version 4.1.3. Samples with a Ct value higher than 27 (n = 5) were eliminated since they are not reliable according to Fluidigm, the owner of the technology (https://www.fluidigm.com, accessed on 21 December 2020). All procedures were conducted as part of the genomics and proteomics service of the Universidad del País Vasco Science Park (Centro de Biotecnología María Goyri).

4.6. Statistics

Results are expressed as raw numbers for categorical variables, as the mean ± standard deviation for continuous normally distributed variables and median (interquartile range) for non-normally distributed variables. To compare unpaired data, Fisher's exact test, Mann–Whitney U test, Student's *t*-test, Kruskal–Wallis, and analysis of variance were applied according to the distribution of variables. Logistic regression analysis was employed for multivariate analysis. Kaplan–Meier analysis was employed for survival analysis with a log-rank test for comparisons between groups. All *p*-values were two-tailed and a p-value < 0.05 was considered significant.

4.7. Bioinformatic Analysis

Bioinformatic analysis was performed at the Statistics and Bioinformatics Unit (UEB) of the Vall d'Hebron Institute of Research (VHIR, Barcelona, Spain). The analyses were carried out with the statistical program "R" (R version 3.6.3 (), Copyright (C) 2021 The R Foundation for Statistical Computing, https://www.R-project.org/, accessed on 29 February 2020). A comprehensive quality control process was applied to assess the suitability of all samples for inclusion in the study. The calculation of relative quantification ($RQ = 2^{-\Delta\Delta Ct}$) was performed according to Livak's method [39]. A principal component analysis (PCA) was performed to describe how the samples are grouped according to the Ct values obtained. Because the variability between genes used as normalizers was low, all were used as housekeeping genes. The geometric mean of the Ct values of the housekeeping genes was obtained as described by Vandesompele et al. [40]. In the process of normalization, the Ct values of each gene were subtracted from the geometric mean value of the two housekeeping genes selected to obtain the ΔCt values. Later, they were used to make comparisons. Spearman's correlation between the expression of each of the genes and tacrolimus levels was performed to select significant genes following criteria of fold change (FC) and statistical significance (FC > 3 and p-value < 0.01).

5. Conclusions

In summary, we evaluated a cohort of patients with an early protocol biopsy and observed that lower tacrolimus through the level at the time of biopsy was associated with interstitial inflammation and a higher expression of rejection-associated transcripts in stable grafts. Cluster analysis allowed the detection of a group of patients who had lower tacrolimus through levels at the time of biopsy, who showed an inflammatory phenotype and displayed a faster decline in renal function in the mid-term. Thus, our results suggest that adjusted immunosuppression during the early months after transplantation favors

better control of the inflammatory response without deleterious effects on renal function in the mid-term. Furthermore, although transcriptomic analysis is not currently widely available in most renal transplant units, its future integration into clinical practice could contribute to improving the management of immunosuppression in renal transplant recipients.

Supplementary Materials: The following are available online at: https://www.mdpi.com/article/10.3390/ijms25063189/s1.

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Informed Consent Statement: All participants in this study provided written informed consent.

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Abbreviations

ABMR	Antibody-mediated rejection.
DSA	Donor-specific HLA antibodies.
FC	Fold change.
FDR	False discovery rate.
eGFR	Estimated glomerular filtration rate by CKD-EPI formula.
IF/TA	Interstitial fibrosis and tubular atrophy.
IF/TA + i	Interstitial fibrosis and tubular atrophy with interstitial infiltrates.
SCR	Subclinical rejection.
TAC-C_0	Tacrolimus trough levels.
TCMR	T cell-mediated rejection.

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5. RESUMEN GLOBAL DE RESULTADOS.

Este trabajo de investigación se origina a partir de un proyecto que tenía como objetivo principal definir la relación entre las distintas formas de monitorización de los niveles de TAC, la inflamación subclínica, la fibrosis intersticial/atrofia tubular y la expresión de genes de rechazo en biopsias de seguimiento en trasplante renal.

La monitorización del injerto renal se fundamenta en la observación de parámetros clínicos en sangre, como la creatinina sérica, la proteinuria y los anticuerpos HLA, así como en la monitorización farmacológica e histológica. Hace más de tres décadas, algunos centros iniciaron programas de biopsias de protocolo, revelando que injertos con función renal estable presentaban cambios histológicos de rechazo, conduciendo a la definición del rechazo subclínico (RSC). Aunque la histología proporciona información significativa, subsisten limitaciones en su interpretación, especialmente en relación con cambios BL y fenotipos incompletos de rechazo mediados por anticuerpos. La incorporación del análisis del transcriptoma puede contribuir a caracterizar mejor estos fenotipos incompletos.

En nuestro primer trabajo titulado: "**A Rejection Gene Expression Score in Indication and Surveillance Biopsies Is Associated with Graft Outcome**", el objetivo era caracterizar la expresión de genes de rechazo en biopsias por indicación y en biopsias de seguimiento con diagnósticos de rechazo subclínico, cambios *borderline* y FI/AT.

Para este primer trabajo se revisaron biopsias realizadas en nuestro centro desde julio del 2015 hasta agosto del 2018, se realizaron 435 biopsias durante este período. En 181 casos se dispuso de un tercer cilindro renal para estudio de genes mediante técnica de microarrays. Para el estudio no se incluyeron pacientes con hallazgos histológicos de recidiva de patología de base, nefropatía diabética, pielonefritis o nefropatía por poliomavirus BK, quedando un total de 161 casos. Finalmente del total de biopsias se escogieron de forma sucesivas las biopsias con diagnóstico histológico normal, rechazo subclínico, rechazo clínico, cambios BL y FI/AT; quedando los grupos conformados por 45 biopsias por indicación y 51 biopsias de seguimiento (total n=96) con los diferentes diagnósticos histológicos.

El primer paso fue utilizar un análisis de componentes principales (ACP) para observar la distribución de los grupos. Se observaron 7 grupos: biopsia normal: Normal-S (n=17); biopsia de rechazo por indicación: REJ-C (n = 12); biopsia de seguimiento con rechazo subclínico: REJ-S (n = 6); cambios *borderline* en biopsias de indicación: BL-C (n = 13); cambios *borderline* en biopsias de seguimiento: BL-S (n = 12); fibrosis intersticial y atrofia tubular en biopsias de indicación: FI/AT-C (n = 20); fibrosis

intersticial y atrofia tubular en biopsias de seguimiento: FI/AT-S (n = 16). El ACP mostró una separación entre las biopsias normales y biopsias con rechazo, quedando entre medio de estas las biopsias con diagnósticos de BL o FI/AT.

Posteriormente, describimos los genes más diferencialmente expresados entre biopsias por indicación y biopsias de seguimiento. Se obtuvo un total de 109 genes expresados más diferencialmente (valor de p <0,01 y fold change (FC)> log 1,75), tras lo cual calculamos la media geométrica de estos genes para definir un score asociado a rechazo (RAG-score). Este score está constituido por genes relacionados con la presentación de antígenos, activación de células T, proteínas citotóxicas, expresión de quimiocinas, transcripciones de células B y células plasmáticas.

La binarización del RAG-score para clasificar las biopsias como positivas o negativas para rechazo (con un punto de corte de positividad ≥5.89), mostró un RAG-score positivo en el 83 % de las biopsias REJ-S, en el 38 % de las biopsias con BL-C, en el 25 % de las biopsias FI/AT-C, el 17 % de las biopsias con BL-S y el 5 % de las biopsias con FI/AT-S. La variabilidad en el RAG-score en estos grupos indica que existen pacientes con y sin señales de rechazo en cada categoría de diagnóstico.

Al realizar la regresión de Cox en el análisis multivariante, observamos que un RAG-score ≥5.89 fue un predictor independiente de los diagnósticos histológicos sobre la evolución del injerto (HR: 3.5, IC 95 %: 1.1–10.9; p = 0.031). Una puntuación RAG positiva, predice el pronóstico del injerto en biopsias de seguimiento y biopsias de indicación, que tienen un fenotipo incompleto de rechazo.

En nuestro segundo trabajo, titulado **"Progression of Interstitial Fibrosis and Tubular Atrophy in Low Immunological Risk Renal Transplants Monitored by Sequential Surveillance Biopsies: The Influence of TAC Exposure and Metabolism"**, exploramos una cohorte de trasplantados renales de bajo riesgo inmunológico (n = 85) tratados con TAC, MMF y esteroides. Disponíamos de biopsias de seguimiento pareadas realizadas a los 3-6 meses y a los 12-18 meses postrasplante. Analizamos las lesiones histológicas y su asociación con parámetros del metabolismo de TAC: niveles de TAC-C_0 al momento de la biopsia; Coeficiente de variabilidad del TAC, tiempo en rango terapéutico y relación C/D de TAC durante el seguimiento.

Observamos que la inflamación intersticial (puntuación i-Banff ≥ 1) en la primera biopsia de seguimiento se asoció con niveles inferiores de TAC-C_0 (OR: 0,69, IC 95 %: 0,50–0,96; p = 0,027). En la segunda biopsia, la inflamación intersticial se asoció

con el tiempo por debajo del rango terapéutico (OR: 1,05 y IC 95 %: 1,01-1,10; p = 0,023). La inflamación en áreas con fibrosis (puntuación i-IFTA ≥ 1) no se asoció con ninguno de los parámetros de evaluación del metabolismo del TAC.

Observamos progresión de la FI/AT en 35 casos (41,2%). Los pacientes con un metabolismo más rápido (relación menor de C/D de TAC), presentaron una mayor progresión de FI/AT, sugiriendo que la nefrotoxicidad de TAC puede contribuir a la progresión de la fibrosis en pacientes estables. Además, se ha descrito una asociación entre los metabolizadores rápidos y una menor función del injerto renal, una mayor incidencia de nefropatía BK y una menor supervivencia del aloinjerto [134-137]. En el análisis multivariante, observamos que tanto la media de C/D de TAC (OR: 0,48; IC 95 %: 0,25–0,92; p = 0,026) como el grado de FI/AT en la primera biopsia (OR: 0,43; IC 95 %: 0,24–0,77, p = 0,005) se asociaron con la progresión de FI/AT.

Por otro lado, aunque la inflamación subclínica se asocia con la progresión de FI/AT [45,138], en nuestro estudio no encontramos esta asociación probablemente en relación con la baja incidencia de inflamación y el bajo riesgo inmunológico de los pacientes [139,140].

Por último, en nuestra tercera publicación titulada: **"Expression of rejection-associated transcripts in early protocol renal transplant biopsies are associated with tacrolimus exposure and graft outcome"**, analizamos la asociación entre la inflamación intersticial, la expresión de genes de rechazo y la implicación de la inmunosupresión en la modulación de estos genes.

En nuestro estudio incluimos tres grupos de pacientes: pacientes con una biopsia de seguimiento normal a los 3 meses de trasplante (grupo I, n = 17); pacientes con biopsia por indicación con rechazo, ya sea RMC o RMA (grupo II, n = 12), y una cohorte de pacientes con biopsia de seguimiento a los 3-4 meses que mostraban diferentes fenotipos histológicos (grupo III, n = 137).

En el grupo II (n = 12), hubo una combinación de casos con RMC (n = 5), RMAca (n = 5) y rechazo mixto (n = 2). En el grupo III, solo biopsias de seguimiento (n = 137) constaban las siguientes categorías histológicas: cambios no específicos (n = 40), RMC subclínico (n = 5), RMA subclínico (n = 3), cambios *borderline* (n = 16), FI/AT sin inflamación intersticial (n = 59) y FI/AT con inflamación intersticial (IF/TA + i) (n = 14).

Para evaluar si hubo una asociación entre la inflamación subclínica, las características del donante/receptor, las variables relacionadas con el trasplante y la

inmunosupresión, comparamos pacientes con inflamación intersticial (i > 0) y pacientes sin inflamación intersticiales (i = 0) en las biopsias de seguimiento. Entre las variables evaluadas, la inflamación subclínica se asoció con un tiempo prolongado de isquemia fría (p = 0,040) y menores niveles TAC-C_0 a la biopsia (p = 0,002).

La expresión génica en los tres grupos de biopsias se analizó en primer lugar mediante análisis de componentes principales. Utilizando RT-PCR en tarjetas microfluídicas para analizar los genes descrito por el Grupo de Banff en 2017 [25]. Encontramos que 111 de los 308 genes evaluados se diferenciaron entre biopsias de seguimiento normales y biopsias por indicación con rechazo. Estos genes estaban relacionados principalmente con RMC (72 genes), RMA (16 genes) y daño tisular (8 genes).

Evaluamos si había una asociación entre los niveles TAC-C_0 en el momento de la biopsia y los 111 genes asociados con el rechazo. En el grupo de I (biopsias de protocolo normal) hubo una estrecha correlación entre niveles de TAC-C_0 a la biopsia y la expresión de los genes IKZF3 y CD2, ambos genes implicados en la regulación y activación de células T [141,142]. Por el contrario, en el grupo II (biopsias por indicación) no hubo correlación entre los niveles de TAC-C0 a la biopsia y la expresión de ningún gen. Finalmente, 19 genes relacionados principalmente con RMC (12 de 19) se correlacionaron con los niveles de TAC-C_0 en el grupo III.

Un análisis no supervisado permitió definir dos clusters de biopsias: uno con todas las biopsias normales y otro con todas las biopsias de rechazo. Las biopsias de seguimiento del Grupo III se distribuyeron en una proporción similar entre ambos grupos (n= 77 en cluster I y n= 60 en cluster 2). Los pacientes que se ubicaron en el cluster 2, presentaron una menor exposición a TAC, mostraron con mayor frecuencia un fenotipo inflamatorio y un descenso más rápido de la función renal a medio plazo.

6. RESUMEN GLOBAL DE LA DISCUSIÓN.

En nuestro **primer trabajo,** observamos que la mayoría de los genes más diferencialmente expresados entre las biopsias normales y las biopsias de rechazo coinciden con conjuntos de genes descritos en trabajos previos [143-145.]

Estos de conjuntos de genes se han obtenido mediante diferentes enfoques. Venner et al. [143], describieron los genes más diferencial expresados entre el RMC y todos los demás diagnósticos, incluido el RMA en biopsia por indicación. Khatri et al. [144], emplearon ocho conjuntos de datos independientes de trasplantes de riñón, hígado, corazón y pulmón para caracterizar los principales genes de rechazo expresados diferencialmente entre órganos. Wang et al. [145], consideraron los genes diferencialmente expresados en el rechazo, cáncer, enfermedades autoinmunes y daño tisular durante la infección. En nuestro estudio, el RAG-score está compuesto principalmente por un conjunto de genes relacionados con la presentación de antígenos, la activación de células T, proteínas citotóxicas, expresión de quimiocinas, transcripciones de células B y células plasmáticas.

Observamos que la puntuación RAG no mostró diferencias entre las biopsias de seguimiento con rechazo subclínico y las biopsias realizadas por rechazo clínico. La puntuación RAG se utilizó de manera binaria para clasificar las biopsias como rechazo o no rechazo. Solo uno de cada seis pacientes con rechazo en las biopsias de seguimiento tuvo una puntuación RAG negativa. Esta observación sugiere que, en el rechazo subclínico, al igual que en el rechazo clínico [83], hay una concordancia razonable entre el diagnóstico histológico y el molecular. Además, esta observación respalda el tratamiento de pacientes con rechazo subclínico [30,39,146.]

En un estudio que evaluó biopsias de seguimiento realizadas a las 6 semanas, se encontró una mayor expresión de genes inflamatorios en biopsias con cambios borderline y rechazo [147]. No obstante, la expresión aumentada de estos genes fue más mayor en paciente con función retrasada del injerto comparado con aquellos con función inmediata. Esto sugiere que, en las biopsias tempranas de seguimiento, la expresión de genes inflamatorios podría también reflejar una respuesta a las lesiones de lesión reparación. En este mismo trabajo, no se encontró asociación entre la expresión de genes inflamatorios y el resultado del injerto a los 2 años. Sin embargo, estudios que evalúan la utilidad del diagnóstico histológico en biopsias tempranas de vigilancia para predecir el resultado del injerto han demostrado que se necesita un seguimiento prolongado para establecer una relación entre la inflamación temprana y los eventos relacionados con el injerto [34,45,46,112].

El RAG-score en biopsias con cambios *borderline* fue mayor en biopsias por indicación que en las biopsias de seguimiento, coincidiendo con resultados de estudios previos [148.] Estos hallazgos sugieren que el deterioro funcional en pacientes con cambios *borderline* podría explicarse parcialmente por una mayor expresión de genes asociados al rechazo. Un RAG-score positivo en biopsias con cambios *borderline* podría indicar un verdadero rechazo, respaldando estudios anteriores que informaron un diagnóstico molecular de rechazo en proporciones similares de casos [81,87].

En cuanto a las biopsias de seguimiento con FI/AT, no mostraron diferencias significativas en comparación con las biopsias normales, lo que sugiere que los injertos estables con FI/AT son inmunológicamente inactivos. Sin embargo, en el 25 % de las biopsias con FI/AT por indicación y un solo caso de las biopsias con FI/AT de seguimiento presentaron un RAG-score positivo.

Aunque la inflamación fue baja en biopsias con FI/AT, ya sea por indicación o seguimiento, una proporción significativa se clasificó como rechazo, según el RAG-score. Esto podría estar relacionado con la sobre expresión de transcripciones inmunes innatas, células presentadoras de antígenos y células T citotóxicas, indicando la activación de mediadores de la señalización de rechazo [83]. Por otro lado, es conocido que la supervivencia del injerto se acorta en las biopsias con FI/AT e inflamación de seguimiento en comparación con las biopsias con FI/AT sin inflamación [43,149].

Finalmente, una puntuación RAG positiva se asoció con la evolución del injerto en los grupos de estudio. Esta asociación fue independiente de la indicación de la biopsia y del diagnóstico histológico. Curiosamente, esta asociación se confirmó cuando se excluyeron del análisis los pacientes con rechazo subclínico.

En cuanto a las limitaciones del estudio, una de ellas fue la falta de una cohorte de validación que confirme la utilidad del RAG-score para predicción de resultados. Sin embargo, en un estudio donde se evaluó la utilidad de un score de rechazo en pacientes con FI/AT sin inflamación, ya sea en biopsias por indicación o de seguimiento, se observó una asociación entre tener un score de rechazo elevado y la supervivencia del injerto [150]. Otra de las limitaciones que observamos fue el reducido tamaño de la muestra, especialmente para evaluar la utilidad del RAG-score en biopsias de seguimiento.

Se han descrito por separado trabajos que relacionan la progresión de FI/AT, mediante biopsias de seguimiento y parámetros de metabolismo del TAC, como niveles bajos de

TAC-C_0 [68], variabilidad intrapaciente [98,151] y relación de C/D de TAC [152]. En nuestro **segundo trabajo** todos estos parámetros se evaluaron en la misma cohorte de trasplantados renales para caracterizar mejor su contribución a la progresión de FI/AT.

No observamos relación entre la progresión de FI/AT con niveles de TAC-C_0, ni tampoco con el TRT, CV-TAC. El principal resultado que obtuvimos fue, que la progresión FI/AT se asoció a la relación C/D de TAC baja entre ambas biopsias. Sin embargo, en nuestra cohorte, los niveles TAC-C_0 fueron relativamente más altos en comparación con otros centros [41], y el tiempo por debajo del rango terapéutico fue menor que en otros estudios, motivo por el cual se entiende que nuestros resultados sean diferentes. La falta de una relación entre los parámetros de metabolismo del TAC y la progresión de FI/AT en nuestro estudio es consistente con los estudios mencionados anteriormente.

Empleamos arbitrariamente una puntuación inflamación (i ≥ 1), como umbral para distinguir entre inflamación y no inflamación. Basamos nuestra decisión en observaciones previas que muestran que una puntuación i ≥ 1 se asocia con una menor supervivencia del injerto, mientras que la tubulitis aislada tiene una influencia menor y controvertida en el resultado [153-155]. Sabemos que la inflamación es un factor conocido de progresión de FI/AT [46,138]. Su incidencia en nuestro estudio fue baja, probablemente debido al bajo riesgo inmunológico de los pacientes [101,139,140], a niveles altos de TAC, y a un mayor tiempo en rango terapéutico, lo que sugiere que incluso en pacientes de bajo riesgo inmunológico que recibieron dosis alta de TAC, la inflamación en áreas sanas puede estar modulada por la exposición al TAC, pudiendo representar un equilibrio entre la respuesta aloinmune y la inmunosupresión.

No encontramos asociación entre la inflamación en áreas cicatriciales y la exposición/metabolismo del TAC. Estudios previos indican que la inflamación en áreas sanas se correlaciona con la presencia de células T citotóxicas, mientras que en áreas cicatriciales se asocia con células B, células plasmáticas, mastocitos y transcripciones de reparación de lesiones [158,159]. Esta diferencia puede ser explicada por el efecto selectivo del TAC sobre las células T activadas [160,161].

La curva farmacocinética en los metabolizadores rápidos se caracteriza por una concentración máxima de TAC (C-max) más alta en comparación con los metabolizadores lentos para alcanzar un TAC-C_0 similar [156,157]. Se ha propuesto que la progresión de FI/AT en estos pacientes puede reflejar nefrotoxicidad por TAC. De

hecho, según nuestros datos, el metabolismo de tacrolimus y no la exposición a tacrolimus contribuyen a la progresión de FI/AT.

El estudio plantea la posibilidad de ajustar el tratamiento de TAC de manera personalizada según la inflamación en áreas sanas, mientras que no habría utilidad en ajustar TAC en inflamaciones en áreas cicatriciales.

Finalmente, el estudio presenta limitaciones, como la falta de biopsias pre-implante para caracterizar la progresión de FI/AT desde el donante hasta la primera biopsia de seguimiento y la ausencia de un estudio farmacocinético completo para evaluar la C-max de TAC en metabolizadores rápidos y lentos, así como la falta de estudio del CYP450.

En cuanto a nuestro **tercer trabajo** analizamos la asociación entre la inflamación intersticial, la expresión de genes de rechazo y la implicación de la inmunosupresión en la modulación de estos genes.

La presencia de inflamación intersticial fue poco común en nuestra cohorte (27.7 %) y estuvo asociada con niveles TAC-C_0 bajos y un tiempo de isquemia fría prolongado, pero no se asoció con un deterioro de la función renal a mediano plazo ni con el desarrollo de *ADEs dn*, esto sugiere que tanto factores inmunológicos como no inmunológicos pueden contribuir a la inflamación subclínica en injertos que funcionan correctamente.

Observamos, al igual que en otros estudios, pocos casos que cumplieron con los criterios de cambios *borderline* (11.7 %), RMC/ RMA (5.8 %) [153]. Mehta et al. [154], efectuaron un estudio en donde se incluyeron pacientes con inmunosupresión libre de esteroides, y observaron una incidencia de cambios BL y RMC 31 % y 20,8 %, respectivamente. Sin embargo, en otro estudio, no encontraron asociaciones entre los niveles TAC-C_0 y la inflamación subclínica cuando los valores promedio de TAC-C_0 eran cercanos a 10 ng/mL[47]. Otros estudios han demostrado que la inflamación mediada por células T detectada en biopsias de seguimiento es secundaria a la isquemia-reperfusión y tiene poca relación con eventos y resultados futuros [147]. La lesión renal aguda (LRA) de la isquemia-reperfusión puede inducir inflamación intersticial y tubulitis [162], lo que conduce a un cuadro histológico que no se distingue del rechazo celular.

La valoración de biopsias mediante histología convencional tiene limitaciones relacionadas con la posibilidad de obtener muestras con baja representatividad y reproducibilidad diagnóstica inter-observador. La heterogeneidad en el análisis transcripcional observada en biopsias con un mismo fenotipo histológico sugiere que diferentes mecanismos inmunopatológicos pueden conducir a un mismo patrón de lesión en el injerto. Por este motivo, se está evaluando incorporar el estudio del fenotipo molecular como complementario a la histología convencional para mejorar la precisión diagnóstica y establecer tratamientos más dirigidos al mecanismo del daño. Esto implica el uso de microarrays en biopsias del injerto para estudiar el fenotipo molecular, lo que ha permitido agrupar diferentes transcritos relacionados con varias patologías y mecanismos biológicos.

Evaluamos el panel de genes descrito por el grupo de Banff en 2017 mediante RT-PCR [25]. Se confirmó la capacidad de discriminación de 111 de los 908 genes evaluados. En la evaluación mediante ACP, observamos que los genes de RMC, expresados en células T efectoras activadas (ADAMDEC1), y los genes que codifican diferentes citoquinas y sus receptores principalmente relacionados con RMC (CCL5, CXCL13 y CXLC9) fueron los más relevantes en el componente 1, mientras que los genes que codifican proteínas de la matriz y transportadores de solutos (COL1A1, SCL22A2 y SCL4A1) fueron los más relevantes en el componente 2.

Identificamos que 19 de estos 111 genes, mayoritariamente asociados con RMC, mostraron correlación con niveles de TAC-C_0, lo cual sugiere que una mayor exposición a tacrolimus favorece un mejor control de la inflamación subclínica. Es interesante destacar que, en el pequeño conjunto de biopsias de seguimiento normal, observamos una correlación estrecha entre niveles de TAC-C_0 y la expresión de 2 de estos 19 genes. El gen IKZF3, presente principalmente en la linfa y el bazo, se encuentra en varios tipos de células inmunitarias, incluyendo células B, células NK, y células T CD4+ y CD8+. Recientemente, se ha demostrado que IKZF3 está regulado positivamente no solo en RMA sino también en muestras de células urinarias de RMC, lo que sugiere que las células B pueden desempeñar un papel más activo RMC de lo que se reconocía anteriormente, tal vez funcionando como células presentadoras de antígenos clásicas [141,142.] También se ha demostrado que la familia CD2 de moléculas coestimuladoras y de adhesión desempeña un papel importante en la ejecución de una respuesta aloinmune, ya que todas las células T la expresan constitutivamente y se regulan positivamente tras el reconocimiento del antígeno.

Posteriormente definimos dos clusters de biopsias. Los pacientes con biopsias de seguimiento que se ubicaron en el cluster 2 recibieron una menor exposición a tacrolimus, mostraron con mayor frecuencia un fenotipo inflamatorio y una disminución más rápida de la función renal a medio plazo. Así, nuestros resultados sugieren que una inmunosupresión más ajustada durante los primeros meses tras el trasplante favorece un mejor control de la respuesta inflamatoria y una mejor preservación de la función renal a medio plazo.

A pesar de la correlación entre ciertos genes y la exposición a TAC, no se encontraron asociaciones significativas entre la expresión génica, la exposición a TAC y la compatibilidad HLA a nivel alélico o molecular. En nuestro estudio, el número de pacientes que desarrollaron *ADE dn* fue muy bajo (2,9 %) y, aunque los pacientes con menor exposición a TAC tendieron a desarrollar *ADE dn* con mayor frecuencia, esta asociación no alcanzó significación estadística.

La principal limitación del estudio es que las asociaciones se basaron en una única determinación de TAC-C_0 al momento de la biopsia, sin una evaluación más detallada de la farmacocinética o farmacodinámica del TAC. Este aspecto podría haber influido en la precisión de nuestras conclusiones sobre la relación entre la exposición a TAC y el pronóstico del injerto.

7. CONCLUSIONES

1. El RAG-score, basado en la expresión de genes de rechazo, demostró una asociación significativa con la presencia de rechazo subclínico en diversas categorías de diagnóstico, incluso en casos de cambios *borderline* y la fibrosis intersticial y atrofia tubular.

2. Un RAG-score positivo se asoció con la supervivencia del injerto de manera independiente al diagnóstico histológico. Esta asociación se mantuvo incluso al excluir pacientes con rechazo subclínico.

3. En nuestro segundo trabajo, la progresión de la fibrosis intersticial y atrofia tubular se asoció con un menor cociente entre la relación concentración/dosis de TAC en biopsias pareadas. Sin embargo, no observamos asociación con la exposición al tacrolimus.

4. En nuestro tercer trabajo, una mayor exposición a tacrolimus durante los primeros meses postrasplante se asoció con mejor control de la inflamación subclínica y preservación de la función renal a medio plazo.

5. En nuestro tercer trabajo, la inflamación intersticial, aunque con baja prevalencia en nuestra cohorte, puede ser influenciada por ambos factores, inmunológicos y no inmunológicos, como la exposición a TAC y la lesión de isquemia reperfusión.

6. Nuestros estudios sugieren la necesidad de una personalización en el manejo del tratamiento inmunosupresor complementado con el diagnóstico molecular y la evaluación del metabolismo del tacrolimus para optimizar los resultados del trasplante renal.

8. LÍNEAS DE INVESTIGACIÓN FUTURAS.

La evaluación histológica de las biopsias de trasplante renal sigue siendo un componente clave en la valoración del injerto. El desarrollo de la clasificación de Banff, utiliza un sistema de consenso basado en reglas para clasificar los casos en categorías de enfermedades según las puntuaciones de las lesiones. En este sistema,

el rechazo mediado por células T (RMC) se diferencia del rechazo mediado por anticuerpos (RMA) principalmente en función de la localización de los infiltrados inmunes (tubulointersticial *versus* microvascular) y en la presencia/ausencia de anticuerpos donante específicos circulantes y el depósito de la fracción C4d del complemento como indicadores de la participación de anticuerpos. Si bien, los diagnósticos moleculares permiten discriminar entre estos principales fenotipos histológicos, la dicotomía entre RMCT y RMA no refleja el mecanismo inmunopatológico subyacente.

La asociación entre los principales fenotipos de rechazo definidos por Banff y el tipo de infiltrado inflamatorio no está bien definido en la actualidad. Estudiar los tipos de células inmunes infiltrantes podría mejorar la comprensión del proceso de rechazo en sus diferentes fenotipos, mejorar la clasificación diagnóstica al incluir más información causal y permitir el desarrollo de tratamientos dirigidos.

Durante los últimos 20 años las tecnologías de transcriptómica, como los microarrays, la secuenciación de ARN (RNA-seq) y la secuenciación de ARN de una sola célula o de un solo núcleo (scRNA-seq o snRNA-seq) han dominado la investigación de perfiles génicos en trasplantes, dando lugar a distintos grupos de investigación. Estas tecnologías han identificado varios paneles de genes diagnósticos para mejorar la detección del rechazo agudo del injerto en las distintas áreas del trasplante (renal, cardíaco, pulmonar y hepático) cuando se utilizan junto con la histopatología tradicional, como los diagnósticos del microscopio molecular, el módulo de rechazo común y el panel Banff de Trasplante de Órganos Humanos (B-HOT). Los diagnósticos moleculares del microscopio y el panel B-HOT han recibido la mayor atención para estudios de validación actuales y futuros.

El perfil transcriptómico en tejidos fijados en formalina e incluidos en parafina (FFPE) ha permitido ampliar las cohortes de validación, incluyendo la correlación entre las puntuaciones Banff y la medición del panel de genes B-HOT en 326 biopsias de trasplante renal FFPE. Este panel pudo detectar evidencia sub patológica de rechazo (antes de las manifestaciones histopatológicas), con inflamación de los capilares peritubulares y anticuerpos específicos del donante, como los principales impulsores de la firma del rechazo mediado por anticuerpos.

En esta línea de investigación nuestro grupo de investigación ha obtenido financiación de un proyecto FIS (PI-01909) con el título “Contribución de la aloreactividad y autorreactividad en el microambiente inflamatorio local en el ABMR en trasplante

renal” con el objetivo de caracterizar la composición del infiltrado inflamatorio de los agregados linfoides en el tejido renal, la formación de tejidos linfoides terciarios (TLTs) y el contenido de células B donante(HLA)-específicas en los distintos fenotipos histológicos de rechazo en trasplante renal y su traducción a través de la biología molecular caracterizando el perfil transcripcional mediante el panel B-HOT y poder definir estos resultados transcripcionales a nivel espacial de los infiltrados inflamatorios con/sin células B donante(HLA)-específicas y su perfil clonal para diferenciar células aloreactivas de autorreactivas.

En esta área de investigación, se ha publicado un trabajo colaborativo con el servicio de Inmunología del Hospital Central de Asturias, en donde se combina **Transcriptómica espacial junto con ARN-secuencial, explorando el uso de esta tecnología para el estudio del rechazo a nivel del injerto renal** [163].

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