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REVIEW

Biomarkers in renal transplantation: An updated review

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Abstract

Genomics, proteomics and molecular biology lead to tremendous advances in all fields of medical sciences. Among these the finding of biomarkers as non invasive indicators of biologic processes represents a useful tool in the field of transplantation. In addition to define the principal characteristics of the biomarkers, this review will examine the biomarker usefulness in the different clinical phases following renal transplantation. Biomarkers of ischemia-reperfusion injury and of delayed graft function are extremely important for an early diagnosis of these complications and for optimizing the treatment. Biomarkers predicting or diagnosing acute rejection either cell-mediated or antibody-mediated allow a risk stratification of the recipient, a prompt diagnosis in an early phase when the histology is still unremarkable. The kidney solid organ response test detects renal transplant recipients at high risk for acute rejection with a very high sensitivity and is also able to make diagnosis of subclinical acute rejection. Other biomarkers are able to detect chronic allograft dysfunction in an early phase and to differentiate the true chronic rejection from other forms of chronic allograft nephropathies no immune related. Finally biomarkers recently discovered identify patients tolerant or almost tolerant. This fact allows to safely reduce or withdrawn the immunosuppressive therapy.

Key words: Renal transplantation; Biomarkers; Genomic; Proteomics; Transplant outcome; Molecular signatures

Core tip: The uses of biomarkers as a non invasive tool instead of renal biopsy in diagnosing transplant renal complications are entering the clinical practice. Progress in genomics, proteomics and all the "omics" fields has allowed the finding of robust, predictive and useful biomarkers. They are modifying our window on transplantation and are allowing us to predict the renal injury earlier because the pathologic process is evident at molecular level before its histological or clinical manifestations. The future is exciting because new international researches and trials are ongoing in this field.

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INTRODUCTION

Kidney transplantation represents the optimal therapeutic tool for patients affected by end-stage renal disease (ESRD). Improvements in immunosuppressive therapy have resulted in a decrease in acute rejections (AR) and have significantly increased graft short-term half life[1]. However, late kidney graft loss remains a major problem and challenge in kidney transplantation[2]. To date, renal function after transplantation is primarily evaluated by serum creatinine measurement and core renal biopsy. The latter is considered the gold standard in transplant evaluation. Nonetheless, both approaches have several drawbacks. Serum creatinine levels increase late in injury and are non-specific for the type of injury. Additionally, the serum level of creatinine is not able to predict or evaluate the progression of chronic injury and as a consequence is not specific or predictive. Similarly, renal core biopsy cannot be used to monitor the progression of injury because it is invasive and cannot be performed serially. Additionally, there are problems and possible biases in evaluating the specimen and the procedure is not completely free of complications. Moreover, the predictive power of renal core biopsy is poor. In fact, in the National Institutes of Health (NIH) clinical trial "Steroid-Free *vs* Steroids-based Immunosuppression in pediatric renal transplantation" (SNSO1) protocol, renal biopsies were unable to measure "hidden" tissue injury in clinically stable patients[3,4]. In addition, using protocol biopsies, Naesens *et al*[5] reported that examination of tissue at the molecular level is able to reveal abnormalities in innate and adoptive immune responses long before those abnormalities appear at the histological level. Clearly, the development of noninvasive reliable and predictive biomarkers for early diagnosis and monitoring of any clinical condition after kidney transplantation is essential for tailored and individualized treatment[6-8].

In studying the entire transplantation process, biological markers may be used throughout all phases, starting from the donor and donor kidney retrieval. In this phase, biomarkers may be useful for predicting short-term outcomes, and the incidence and severity of delayed graft function (DGF).

The most studied and used biomarkers are those related to the diagnosis and the identification of different aspects of subacute and acute kidney rejection. In addition, biomarkers able to differentiate true chronic rejection (CR), which is immunologically mediated, from the so-called "chronic allograft dysfunction" (CAD), are important because the treatments are different. Indeed, recently, mining the human urine proteome for monitoring renal transplant injury, Sigdel *et al*[9] found urinary peptides specific for AR, urinary peptides specific for chronic allograft nephropathy (CAN) and urinary peptides specific for BK virus nephropathy (BKVN).

Finally, relevant markers are those associated with tolerance, as these markers might allow for decreasing immunosuppressive treatment, withdrawing or discontinuing any immunosuppressant and monitoring the effects of such measures.

In this review, we describe the principal characteristics of current biomarkers, their power and limitation, the principal sources and their relevance in different clinical settings post renal transplantation.

RESEARCH METHODOLOGY

For this review, we have analyzed the available papers on biomarkers in renal transplantation. A literature search was performed using PubMed (NCBI/NIH) with the search words renal transplantation, biomarkers, genomic, proteomics, transplant outcome, molecular signatures. Firstly, papers published in the last three years were examined, then we proceeded in a backward way and also studies published previously have been included. Studies currently under way were searched for in "clinical trial.gov" and the European EUDRACT register. Only randomized clinical trials (RCTs) active and enrolling patients have been selected.

DEFINITION AND PRINCIPAL CHARACTERISTICS OF THE BIOLOGICAL MARKERS

In addition to clinical markers and pathological markers, monitoring of the outcome of a clinical process may be performed using biological markers (biomarkers). A NIH working group recommended the following terms and definitions[10]: A biomarker is a characteristic that is objectively measured and evaluated as an indicator of a normal biological process, pathogenic process or pharmacological response to a therapeutic intervention.

Principal applications of biomarkers are as follows: (1) diagnosis or identification of patients affected by a disease or an abnormal condition; (2) staging of the severity or extent of a disease; (3) prognosis of a disease; and (4) prediction and monitoring of a clinical response to an intervention.

Table 1 clarifies both the definition and the principal characteristics of the biomarkers and the technologies involved[11]. A variety of innovative technologies, ranging from genomics, proteomics, peptidomics, antibodyomics, microbiomics and metabolomics, among others, all referred to as "omics", have emerged in medical fields, to generate new biomarkers[12].

Genomics refers to the study of the genome, and epigenomics is the study of the complete set of epigenetic modifications of the genetic materials of a cell. Transcriptomics is the study of the set of all messenger

Table 1 Definition and principal characteristics of biomarkers

Biomarker	A characteristic objectively measured as an indicator of a biological process or a response to a pharmacological intervention
Proteomics	The systematic analysis of proteins for their identity, quantity and function
Genomics	The study of the genome for estimating the risk for an individual to develop a disease
Transcriptomics	The study of expression patterns of all gene transcript
Metabolomics	The quantitative analysis of all the metabolites of a specific biological sample

Table 2 Biomarker candidates in the context of ischemia reperfusion injury and delayed graft function

Symbol	Gene description	Cytoband
ACTA2	Actin, alpha 2, smooth muscle, aorta	*10q23.31*
UMOD	Uromodulin	*16p12.3*
LGALS3	Lectin, galactoside-binding, soluble, 3	*14q22.3*
SAT1	Spermidine/spermine N1-acetyltransferase 1	*Xp22.11*
HAVCR1	Hepatitis A virus cellular receptor 1	*5q33.3*
CXCL1	Chemokine (C-X-C motif) ligand 1	*4q13.3*
ANXA2	Annexin A2	*15q22.2*
S100A6	S100 calcium binding protein A6	*1q21.3*
CYR61	Cysteine rich angiogenic inducer 61	*1p22.3*
S100B	S100 calcium binding protein B	*21q22.3*
AMBP	Alpha-1-microglobulin/bikunin precursor	*9q32*
LCN2	Lipocalin 2	*9q34.11*
C3	Complement component 3	*19p13.3*
FABP1	Fatty acid binding protein 1, liver	*2p11.2*
ATF3	Activating transcription factor 3	*1q32.3*
NTN1	Netrin 1	*17p13.1*
ENG	Endoglin	*9q34.11*
GUCY2G	Guanylate cyclase 2G	*10q25.2*
BID	BH3 interacting domain death agonist	*22q11.21*
BCL2	B-Cell CLL/lymphoma 2	*18q21.33*
BAX	BCL2 associated X protein	*19q13.33*
PTGS2	Prostaglandin-endoperoxide synthase 2	*1q31.1*
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif 1	*21q21.3*
CDKN1A	Cyclin dependent kinase inhibitor 1A	*6p21.2*

RNA molecules in a population of cells, whereas proteomics is the systematic analysis of proteins with regard to their identity, quantity and function. Metabolomics is the study of all chemical processes involving metabolites.

Overall, the principal characteristics, challenges and limitations of the biomarkers applied in renal transplantation are as follows: (1) Sensitivity, specificity, positive and negative predictive values and receiver operating characteristics curves (ROC) of biomarkers are essential for assessing their clinical utility; (2) noninvasive candidate biomarkers principally include mRNA transcripts, lymphocyte phenotype markers, chemokines, microRNA (miRNA) and donor-specific antibodies; (3) robust validation studies and assay standardization are needed to identify new biomarkers; and (4) biomarker validations is challenging because of interindividual variations as well as interlaboratory and interplatform variability[13-15].

The main sources of biomarkers in renal transplantation are serum, urine, peripheral blood lymphocytes and tissue.

BIOMARKERS OF ISCHEMIA-REPERFUSION SYNDROME AND DGF

Ischemia reperfusion injury (IRI) is an unavoidable step occurring after kidney transplantation and may influence both short-term and long-term graft outcomes. Clinically, IRI may be associated with delayed DGF, graft rejection, CR and CAD[16]. The degree of IRI is related to several factors that may occur in the donor, during organ storage and in the recipient[17]. The increasing use of extended criteria donors and the use of organs recovered from non-heart-beating donors (NHBDs) represent an increased risk of severe IRI. Clearly, understanding the factors that potentially lead to severe IRI allow for stratifying the risk to the recipients and for a prompt diagnosis of IRI, enabling the adoption of possible therapeutic measures of prevention and treatment. Identification of biomarkers for IRI may assist in this effort.

Table 2 report a number of biomarkers candidates within the context of IRI and DGF. Such biomarkers have been studied pre or post-transplantation[18].

Pre-transplant biomarkers for IRI and DGF

A number of molecules expressing tubular or vascular damage in the donor organ are associated with the incidence and severity of IRI. In turn, the severity of IRI conditions the incidence of DGF[19,20] and graft survival is strictly related to the incidence of DGF[21].

Proteomic studies: Holmen *et al*[22] documented the predictive value of urinary neutrophil gelatinase-associated lipocalin (uNGAL) levels for prolonged DGF. This finding has been confirmed by a study of Reese *et al*[23]. A predictive value of donor uNGAL, urinary kidney injury molecule 1 (uKIM-1) and urinary fatty acid protein binding protein (u-FABP) for DGF was recently documented by a study of Koo *et al*[24].

Other studies documented the association of recipient pretransplant levels of different cytokines as the soluble interleukin 6 receptor (sIL-6R)[25] and the low soluble gp130 with post-transplant DGF.

Recently, Nguyen *et al*[26] measuring tumour necrosis factor receptor 2 (TNFR-2) expressed on circulating T reg cells documented that recipient peripheral blood T reg is a pre-transplant predictor of DGF.

Genomic studies: Several studies have investigated the pre-transplant up-regulation of genes possibly associated with IRI and DGF. One of the main limitations in identifying these molecules as a real marker of inflammation and a potential therapeutic target is the lack of causal proof.

In two different studies Schwartz *et al*[27,28] documented that the expression of tubular epithelial cell adhesion molecules was predictive of post-transplant DGF and, similarly, that the lack of up regulation of anti apoptotic genes as B cell lymphoma 2 (*Bcl-2*) and B cell lymphoma extralarge (*Bcl-xl*) in donor kidneys was associated with DGF. More recently, Kaminska *et al*[29] studying the pre-transplant intragraft expression of 29 genes, found that lipocalin-2 (*LCN)* or *NGAL* were related to DGF.

Hauser *et al*[30] and Kainz *et al*[31] studied the expression of 48 genes associated with DGF in pretransplant biopsies and found an up-regulation of genes related to complement and to metabolic and immune pathways. More recently McGuinnes *et al*[32] found that an elevated expression of cyclin-dependent kinase inhibitor 2A (CDKN2A*)* correlated with high DGF incidence.

A recent trial was conducted (ISRCTN78828338) to verify whether steroid pretreatment of the deceased organ donor was able to reduce the incidence of IRI and DGF.

Genomic analysis showed suppressed inflammation and immune response in kidney biopsies from deceased donors who received corticosteroids. Among the proteins encoded by these identified genes, steroids significantly reduced FK506-binding protein 5 (*FKBP5*), ring finger protein 186 (*RNF186*), TSC22 domain family member 3 (*TSC22D3*), Phospholambam (*PLN*), Solute carrier family 25, member 45 (*SLC25A45*), Small G protein signaling modulator 3 (*SGM3*) and Sushi domain-containing protein 3 (*SUSD3*). However, two studies related to the trial[33,34] concluded that such inflammation suppression did not reduce the incidence or duration of post-transplant DGF in allograft recipients; taken together, the studies documented that steroid pretreatment of organ donors did not improve outcomes after kidney or liver transplantation.

Post-transplant biomarkers for IRI and DGF

Proteomic and genomic studies: Liangos *et al*[35] conducted a study on patients affected by DGF and documented an association between KIM 1 levels and disease severity.

Several studies have examined the utility of determining serum or urinary levels of NGAL in predicting DGF after renal transplantation.

Experimental and clinical models have documented that urinary biomarkers such as uNGAL, uKIM-1, uIL-18 and u-FABP are specific for acute kidney injury (AKI) and/or IRI[36,37]. Several recipient urinary biomarkers are also reported to be related to graft dysfunction[38-42].

More recently, two studies documented that urinary clusterin and IL-18 allow predicting DGF within 4 h after transplantation[43]. Similarly, NGAL reflects the entity of renal impairment, representing a useful biomarker and an independent risk factor not only for DGF but also for long-term graft dysfunction[44].

A study by Hall *et al*[45,46] showed by multivariate analysis that elevated urinary levels of NGAL or IL-18 were able to predict DGF, with a ROC of 0.82.

Other studies[47,48] documented that high urinary levels of NGAL soon after transplantation are found in patients with AKI, in particular when AKI is due to AR. In a more recent meta-analysis involving 16500 critically ill patients or following cardiac surgery, elevated plasma or urinary levels of NGAL were associated with AKI but not related to rejection[49]. Finally, in a recent review[50], high urinary or serum NGAL levels were found to serve as a predictor of DGF and were associated with reduced graft function at 1 year.

To date several studies have investigated the role of miRNAs as biomarkers of DGF. miRNAs, short endogenous non-coding RNAs that inhibit gene expression, play a fundamental role in DNA and protein biosynthesis. Some studies found that miRNAs contribute to both the induction and progression of chronic kidney disease (CKD)[51]. miRNAs also represent novel therapeutic targets for CKD and for various complications after renal transplantation[52]. A role in the pathogenesis of post-transplant DGF was found for 2 miRNAs: miR-182-5p and mi-21-3p[53]. The same author found high levels of secretory leukocyte peptidase inhibitor (SLPI) in serum and urine proteome of patients affected by AKI post-transplantation as well as a novel miRNA, miR-182-5p[53].

In summary, miRNAs have a potential role as new biomarkers in all phases of kidney transplantation, even though most of the studies concerning IRI thus far have been conducted on mice[54].

Overall the use of biomarkers, though relevant, has several limitations in the field of IRI. First most studies have been conducted on mice, and their translation to humans is questionable. Second, a proof of cause is lacking, and the only study performed with regard to reducing markers of inflammation failed to report a reduction in IRI incidence and severity. Third, a gold standard for comparison, such as renal biopsy, is lacking.

BIOMARKERS FOR ACUTE REJECTION

For acute rejection also pretransplant biomarkers have been described.

Pre-transplant biomarkers for acute rejection

The most investigated pre-transplant serum biomarker has been the soluble form of CD30 (sCD30). sCD30 is a glycoprotein expressed on human $CD4^+CD8^+$ T cells that secretes Th2-type cytokines[55]. sCD30 reflects those recipients who will generate an alloimmune response against a grafted kidney. Weimer *et al*[56] documented that sCD30 was a predictor of a poor graft outcome. Other studies highlighted that more often such poor outcome was related to a higher incidence of AR[57-61].

Other studies[62,63] found that recipients with increased levels of C-X-C motif chemokine ligand 10 (CXCL10), an interferon induced chemokine associated with Th1 immune response have higher incidence transplant failure due to a higher AR incidence. Similar findings have been reported for C-X-C motif chemokine ligand 9 (CXCL9)[64].

Using systematic application of interferon-gamma (IFN-gamma) enzyme linked immunospot (ELISPOT) assay, different studies documented that the pretransplant frequency of donor specific IFN-gamma-producing cells correlates with AR among recipients of cadaveric kidney allograft[65-68].

Post-transplant biomarkers for acute rejection

Based on the studies of Naesens *et al*[5] and Sigdel *et al*[9], including genomic and proteomic studies, there are two important points concerning acute and CR, both from genomic and proteomic studies. First, genomic studies have confirmed that smoldering tissue immune activation increases over-time after transplantation and drives progressive CAN independently from AR episodes. Second, the same genomic studies reported that molecular injury in CAN and AR is similar. There is a "so-called" threshold effect for AR, and in the clinical phase of AR, the molecular injury is the same as that found in CAN, though at a higher level. These results were confirmed by urinary proteomic studies. It is therefore important to determine a sensitive and robust biomarker for differentiating AR from other forms of CAD.

Several unbiased plasma and urine proteomic studies have revealed molecules associated with AR. Cohen Freue *et al*[69] found that 7 proteins were up-regulated in the plasma of patients with acute rejection, including connectin (TTN), lipopolysaccharide-binding protein (LBP), peptidase inhibitor 16 (PI16), complement factor D (CFD), mannose-binding lectin (MBL2), recombinant SERPINA10 protein (SERPINA 10) and beta 2 microglobulin (B2M). Using urine samples, Sigdel[70] found proteins related to major histocompatibility complex (MHC) antigens and the complement cascade. Proteins such as uromodulin, serpin peptidase inhibitor, clade F member 1 (SERPINF1) and CD44 were further validated by enzyme-linked immunosorbent assay (ELISA) and Wu *et al*[71] reported 66 proteins in plasma associated with AR, including nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 1 (STAT1) and STAT3. In addition, Loftheim *et al*[72] reported growth-related proteins as Insulin-like growth factor-binding protein (IGFBP7), Vasorin, epidermal growth factor (EGF) and Galactin-3 binding protein (Gal-3BP) to be up-regulated in urine during AR.

Finally, in a recent study, Sigdel *et al*[73] identified and validated by ELISA three urine proteins: Fibrinogen beta (FGB), fibrinogen gamma (FGG) and HLADRB1 during AR. Proteins related to BKVN and CAN were also identified in the same study. All these studies are listed in Table 3.

Other selected studies of biomarkers specific for AR were recently reported by Lo *et al*[7]. Granzyme B (GZMB), perforin (PRF1) and Fas Ligand (FASLG) mRNA are elevated in peripheral blood and tissue[74]. GZMB and PRF1 mRNA are also elevated in the urine of patients with AR[75]. By investigating mRNAs in urinary cells, elevated levels of gene signature of tumor necrosis factor (TNF) receptor superfamily member 4 (*TNFRSF4),* TNF ligand superfamily member 4 (*TNFSF4),* and programmed cell death protein 1 (*PDCD1*) were found in another study[76]. The multicenter CTOT 04 trial reported a urinary three- gene signature of 18S ribosomal RNA of CD3ε mRNA, interferon inducible protein 10 (CXCL10) mRNA and 18S rRNA in patients with biopsy-confirmed acute cellular rejection[77]. CTOT-01 study[78] also revealed elevated levels of urinary CXCL9 mRNA as the best predictor of AR and the authors of this study[78] concluded that low urinary CXCL9 could be used as a biomarker to identify transplant recipients at low risk for immunological events[79]. The findings of the CTOT-01 study represent important news in the field of biomarkers and immunological events in transplantation. Nonetheless, the following open questions remain: (1) whether urinary CXCL9 can be used to decrease indication rates for performing renal biopsy; (2) whether CXCL9 is an adequate tool to distinguish between rejection and injury not immunologically related; and (3) whether the absence of urinary CXCL9 might help to identify the subset of patients whose immunosuppression may be reduced without risks. In a Canadian study[80], the urinary CXCR3 chemokine receptor was shown to be the most promising candidate for detecting subclinical inflammation. This receptor decreases after successful treatment and has a predictive value for detecting subsequent CAN.

In a recent review of urine proteomics[81] , several urine biomarkers were correlated with allograft injury, including CXCL9, CXCL10, C-C motif chemokine ligand 2 (CCL2), NGAL, IL-18, cystatin C, KIM1, T-cell immunoglobulin and mucine domains-containing protein 3 (TIM3). The review also highlighted the aforementioned findings of the CTOT-01 study[78]. In a very recent study[82], four new proteins were found to be related to AR: Alpha-1-antitrypsin (A1AT), alpha 2 antiplasmin (A2AP), serum amyloid A (SAA) and apolipoprotein CIII (APOC3).

miRNAs play critical roles in the modulation of innate and adaptive immune responses. Sui *et al*[83] found 20

Table 3 Unbiased proteomic studies for acute rejection

Ref.	Biomarker candidate	Sample type	Sample numbers	Outcome
Freue *et al*[69]	TTN, LBP, CFD, MBL2, SERPINA10, AFM, KNG1, LCAT, SHBG	Plasma	32	AR
Sigdel *et al*[70]	UMOD, PEDF, CD44	Urine	60	AR
Wu *et al*[71]	NF-κB, STAT1, STAT3 and 63 other proteins	Plasma	13	AR
Loftheim *et al*[72]	IGFBP7, VASN, EGF, LG3BP	Urine	12	AR
Sigdel *et al*[73]	HLA-DRB1, FGB, FGA, KRT14, HIST1H4B, ACTB, KRT7, DPP4	Urine	154	AR

AR: Acute rejection; TTN: Titin; LBP: Lipid binding protein; MBL2: Mannose binding lectin 2; SERPINA 10: Protein Z-dependent protease inhibitor; AFM: Atomic force microscopy; KNG1: Kininogen1 protein; LCAT: Lecithin–cholesterol acyltransferase; SHBG: Sex hormon binding protein; UMOD: Uromodulin; PEDF: Pigment epithelium derived factor; NFκB: Nuclear factor kappa B; STAT1: Signal transducer and activator of transcription 1; STAT3: Signal transducer and activator of transcription 3; IGFBP7: Insulin like growth factor binding protein 7; VASN: Vasorin; EGF: Epidermal growth factor; LG3BP: Galectin-3-binding protein; FGB: Fibrinogen beta chain precursor; FGA: Fibrinogen alpha chain precursor; KRT14: Keratin14; HIST1H4B: Histone cluster 1 H4 family member b; ACTB: Actin beta; KRT7: Keratin 7; DPP4: Dipeptidil-peptidasi 4.

Table 4 Selected promising molecules and pathways evaluated as biomarkers in acute rejection[7]

Biomarker	Sample (assay method)	Patients/ samples	Rejection/no rejection	Sensitivity/ specificity (%)	PPV/NPV(%)	AUC
Granzyme B, perforin and FasL[74]	PBL (PCR)	25/31	11/20	100/95	100/95	NA
FOXP3[88]	PBL, urine (PCR)	65/78	20/58	94-100/ 95/100	94-100/ 95/100	0.95-1.00
Granzyme B, perforin[75]	Urine (PCR)	85/151	24/127	79-83/77-83	NA	NA
OX40, OX40L, PD-1 and FOXP3[76]	Urine (PCR)	46/46	21/25	95/92	NA	0.98
CD3ε,CXCL10, 18S rRNA[77]	Urine (PCR)	485/4300	43/1,70	79/78 (71/72)	NA	0.85 (0.74)
TIM-3[81]	PBL, urine (PCR)	115/160	65/95	87-100/95-100	87-100/93-100	0.96-1.00
CXCL9, CXCL10[78]	Urine (multiplex bead assay)	156/156	25/131	80-86/76-80	NA	0.83-0.87
CXCL9 mRNA and protein[79]	PBL, urine (PCR, ELISA, SELDI-TOF-MS	280/2770	37/113	66.7-85.2/ 79.6/80.7	61.5/67.6/83-92	0.78-0.85
miR-142-5p miR-155 miR-223[83]	Biopsy sample (PCR)	32/33	12/21	92-100/90-95	NA	0.96-0.99
miR-210[85]	Urine (PCR)	81/88	68/20	52/74	NA	0.7
IFNγ-producing memory T cells[89]	PBL (ELISPOT)	23/23	12/10	80/83	NA	0.8

All the studies include a validation set. PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the curve; PBL: Peripheral blood lymphocytes; PCR: Polymerase chain reaction; NA: Not available; PD-1: Programmed death 1; CXCL10: Interferon-inducible cytokine IP-10; 18S rRNA: 18S ribosomal RNA; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; CXCL9: C-X-C motif chemokine 9; ELISA: Enzyme-linked immunosorbent assay; SELDI-TOF-MS: Surface-enhanced laser desorption/ionization time-of-flight MS; miRNA: microRNA; IFNγ: Interferon gamma; ELISPOT: Enzyme-linked immunoSpot.

miRNAs in AR samples, 8 of which were up-regulated and 12 down-regulated. These findings were confirmed in another study by Anglicheau *et al*[84]. Lorenzen *et al*[85] demonstrated a specific role for urinary miR-210, decreasing during AR but normalizing after successful treatment.

Studies of miRNA in peripheral blood cells (PBCs) are also emerging. For example, Betts *et al*[86] in a small study found miR-223 and miRNA 10a to be significantly reduced during AR. In another study Grigoryev *et al*[87] found that inhibition of miR-155 and miR-221 is associated with T cell proliferation, whereas miR-142-3p is associated with tolerant kidney allograft recipients.

Other studies have documented that the level of forkhead box P3 (FOXP3) mRNA in urinary cells is higher in patients with biopsy-confirmed AR[88]. In the same study, the association between low FOXP3 mRNA and high serum creatinine predicted a poor allograft outcome.

T lymphocytes are also being studied as markers of AR. ELISPOT is the best tool for evaluating T lymphocyte phenotypes, and more reliable results are obtained by studies detecting the quantity of IFNγ-producing T cells after stimulation with donor antigens[89]. The Reprogramming the Immune System for Establishment of Tolerance (RISET) consortium has also demonstrated the value of the IFNγ assay[90]. All these studies are reported in Table 4.

Finally, donor-derived cell-free DNA (ddcfDNA) may be detected in the recipient's blood and urine[91]. Indeed, García Moreira *et al*[92] documented an increase in ddcfDNA during AR.

However, the specificity of this finding is questionable because Sigdel *et al*[93] found that ddcfDNA in urine was also present in AR and in BKVN. Additionally, urinary ddcfDNA may be present in cases of pyelonephritis[94]. Thus, the usefulness of ddcfDNA in detecting AR remains questionable.

Genomic studies for acute rejection: With the evolution of array technologies, new insight is surfacing and

Table 5 Seventeen genes involved in the study kidney solid organ response test

Symbol	Gene name	Cytoband
Genes derived from the NIH SNSO1 study		
DUSP1	Dual-specificity phosphatase 1	*5q35.1*
NAMPT	Nicotinamide phosphoribosyltransferase	*7q22.3*
PSEN1	Presenilin 1	*14q24.2*
MAPK9	Mitogen-activated protein kinase 9	*5q35.3*
NKTR	Natural killer cell triggering receptor	*3p22.1*
CFLAR	CASP8 and FADD like apoptosis regulator gene	*2q33.1*
IFNGR1	Ligand binding chain of the gamma interferon receptor gene	*6q23.3*
ITGAX	Integrin alphaXchain protein encoding gene	*16p11.2*
RNF130	Ring finger motif encoding gene	*5q35.3*
RYBP	RING1 and YY1 binding protein encoding gene	*3p13*
Genes added to improve the accuracy of kSORT		
CEACAM4	Carcinoembryonic antigen related cell adhesion molecule 4	*19q13.2*
EPOR	Erythropoietin receptor encoding gene	*19p13.2*
GZMK	Granzyme K encoding gene	*5q11.2*
RARA	Retinoic acid receptor encoding gene	*17q21.2*
RHEB	Ras homolog enriched in brain encoding gene	*7q36.1*
RXRA	Retinoic X receptor alpha encoding gene	*9q34.2*
SLC25A37	Solute carrier family 25 number 37 encoding gene	*8p21.2*

The 17 gene set was selected in 143 samples for acute rejection classification and predicted AR up to 3 mo prior to detection by the current gold standard (biopsy). kSORT: Kidney solid organ response test; SNSO1: Steroid-Free *vs* Steroid-Based Immunosuppression in Pediatric Renal (Kidney) Transplantation.

genomic studies are being applied to detect AR[95].

In the CTOT-04 study, Suthanthiran *et al*[77] found an AR diagnostic three gene signature: CD3ε, IP-10 and 185r RNAs[78].

Flechner *et al*[96] in a small study reported that several genes in peripheral blood lymphocytes (PBLs) and in kidney biopsies are able to characterize patients with AR. These genes are related to immune inflammation, transcription factors, cell growth and DNA metabolism.

The NIH SNSO1 randomized study collected human blood and graft biopsies from 367 patients from 12 United States pediatric transplant programs. The genes revealed by microarray were subsequently validated by quantitative polymerase chain reaction (qPCR). A five-gene set [dual specifity phosphatase 1 (*DUSP1*), nicotinamide phosphoribosyltransferase (*PBEF1*), presenil 1 gene (*PSEN1*), mitogen-activated protein kinase 9 gene (*MAPK9*) and natural killer cell-triggering receptor gene (*NKTR*)] was able to identify patients affected by AR with high accuracy (ROC AUC = 0.955), though the addition of five other genes known to be involved in AR did not improve the accuracy[97,98]. Kurian *et al*[99] reported 200 genes possibly related to AR, with ROC values ranging from 76% to 95%. However, the number of patients enrolled was rather small, and the findings need to be verified.

The assessment of AR in renal transplantation (the AART study) involved 436 adult/pediatric renal transplant patients from eight transplant centers in the United States, Spain and Mexico, and the kidney solid organ response test (kSORT) was used to detect renal transplant patients at high risk for AR in the AART study[100]. A 43 rejection-gene set related to AR was identified by genome microarray analysis of biopsies and blood from patients enrolled in the study[97,101].

Ten of these genes were also found in the NIH SNSO1 study[97]. Utilizing different statistical methods for improve accuracy in diagnosing AR, seven additional genes were added in the kSORT study. All these genes are shown in Table 5.

The kSORT results using a 17-gene set had very high sensitivity (AUC = 0.944), and these results were validated in several ways, such as in adult *vs* pediatric recipients, in samples collected from different sites and in samples across different ages and settings.

Overall, kSORT performance was similar among different cohorts (training set, validation set, cross-validation set (Table 6).

kSORT was also able to predict subclinical acute rejection (scAR) alone or in combination with the IFNγ ELISPOT. In the evaluation of subclinical acute rejection prediction study (ESCAPE)[102], both techniques were applied in renal transplant patients with protocol biopsies at 6 mo. The kSORT assay documented high accuracy in predicting both sub clinical antibody-mediated rejection (scABMR) and sub clinical T cell-mediated rejection (scTCMR). ELISPOT was also predictive for scTCMR but less specific in diagnosing scABMR. The predictive probabilities for diagnosing both scABMR and scTCMR were higher when combining the assays, with an AUC > 0.85.

A different approach for identifying acute rejection genes is to employ meta-analysis of eight independent datasets from four different organs (heart, kidney, liver and lung allograft), and a common rejection module (CRM) consisting of 11 genes significantly over-expressed in AR was thus identified[103]. These genes are presented in Table 7.

Table 6 Performance of kidney solid organ response test in the acute rejection in renal transplantation AART143, AART124, and AART100 cohorts

	kSORT predictions					
	AART143 (training set)		AART124 (validation set)		AART100 (cross-validation set)	
	AR	No AR	AR	No AR	AR	No AR
Real results						3
AR	39	8	21	2	36	43
No AR	9	87	1	100	3	
Sensitivity (95%CI)	82.98% (69.19%-92.35%)		91.30% (71.96%-98.38%)		92.31% (79.13%-98.38%)	
Specificity (95%CI)	90.63% (82.95%-95.62%)		99.01% (94.61%-99.97%)		93.48% (82.1%-96.63%)	
PPV (95%CI)	81.25% (68.06%-89.81%)		95.46% (78.20%-99.19%)		93.21% (79.68%-97.35%)	
NPV (95%CI)	91.58% (84.25%-95.67%)		98.04% (93.13%-99.46%)		93.48% (82.45%-97.76%)	
AUC (95%CI)	0.94 (0.91-0.98)		0.95 (0.88-1.00)		0.92 (0.86-0.98)	

kSORT: Kidney solid organ response test; AART: Assessment of acute rejection in renal transplantation; AR: Acute rejection; PPV: Positive predictive value; NPV: Negative predictive value; AUC: Area under the curve.

Table 7 Eleven genes overexpressed in the common rejection module[103]

Symbol	Gene name	Cytoband
BASP1	Brain abundant membrane attached signal protein 1	*5p15.1*
CD6	CD6 molecule	*11q12.2*
CXCL10	C-X-C Motif chemokine ligand 10	*4q21.1*
CXCL9	C-X-C Motif chemokine ligand 9	*4q21.1*
INPP5D	Inositol polyphosphate-5-phosphatase D	*2q37.1*
ISG20	Interferon stimulated exonuclease gene 20	*15q26.1*
LCK	LCK protooncogene, SRC family tyrosine kinase	*1p35.2*
NKG7	Natural killer cell granule protein 7	*19q13.41*
PSMB9	Proteasome subunit beta 9	*6p21.32*
RUNX3	Runt related transcription factor 3	*1p36.11*
TAP1	Transporter 1, ATP binding cassette subfamily B member	*6p21.32*

These genes were overexpressed in acute rejection across all transplanted organs and could diagnose acute rejection with high specificity and sensitivity.

In a study on the kidney, the 11-gene qPCR CRM score (tCRM) was found to be significantly increased in AR, with the greatest significance for CXCL9 and CXCL10[104]. Additionally, the tCRM score correlated with the extent of AR lesions and was predictive of CAD. In the already mentioned paper by Li *et al*[97], 8 genes were found by qPCR to be overexpressed in AR (*CFLAR*, $P = 0.0016$; *DUSP1*, $P = 0.0013$; *IFNGR1*, $P = 0.0062$; *ITGAX*, $P = 0.0011$; *PBEF1*, $P = 0.00008$; *PSEN1*, $P = 0.00007$; *RNF130*, $P = 0.0459$; and *RYBP*, $P = 0012$) and 2 genes were underexpressed (*MAPK9*, $P = 0.0006$; *NKTR*, $P = 0016$).

More recently[105], PCR measurement of the above gene set was evaluated in the urine of transplanted patients with acute allograft dysfunction; only 5/11 genes were highly significant at the time of rejection, and in a validation cohort, the urine common rejection module (uCRM) score for AR had an AUC of 0.961. However, in another study, the uCRM score was found to be elevated in other kidney injuries, such as acute tubular necrosis (ATN) and BKVN.

In summary, the suspicion of AR in kidney transplantation may be assessed by both proteomic and genomic biomarkers. Principal limitations appear to be the specificity of the biomarkers, as many of them are common with CAN and other forms of chronic nephropathies such as the related condition BKVN.

In the last years, genomic analyses are becoming more specific, and relevant progress has been made by kSORT applied to AART study. Unifying databases derived from studies on acute rejection of other organs such as the liver, lung and heart have allowed for realization of a common rejection module from which new genes specific for kidney rejection can be found.

BIOMARKERS FOR CAD

The term CAD has replaced the term CAN because the latter has been used too broadly, preventing identification of true CR and other aetiologies of chronic dysfunction, such as drugs and viruses, not related to immunological causes. Two main concerns are associated with the identification of non-invasive biomarkers of CAD. First several proteomic and genomic studies[7,9] have found that the molecular mechanisms responsible for acute and CR may be extremely similar and that differentiation should be principally based on the so-called "threshold effect". As a consequence, identification of biomarkers

Table 8 Analysis of pooled urine proteins collected from patients with acute rejection, BK virus nephropathy, and chronic allograft nephropathy when compared to STA urine with the criteria of > 1.5 fold change of each transplant injury phenotype (acute rejection, BK virus nephropathy, and chronic allograft nephropathy), compared to STA pooled urine and with a *P*-value of ≤ 0.05[131]

Increased in AR	Increased in BKVN	Increased in CAN
HLA-DRB1, FGB, FGA, FGG, KRT14, HIST1H4B, KRT7, DPP4	KRT18, SUMO2, STMN1, CFHR2, KRT8, KRT19, RPL18, KRT75, FAM3C, HIST1H2BA	CALR, FAM151A, SERPINA2P, FAM3C, DAG1, KITLG, LUM, FABP4, AGT, LRG1

AR: Acute rejection; BKVN: BK virus nephropathy; CAN: Chronic allograft nephropathy; FGB: Fibrinogen beta chain; FGA: Fibrinogen alpha chain; FGG: Fibrinogen gamma chain; KRT14: Keratin 14; HIST1H4B: Histone cluster 1 H4 family member b; KRT7: Keratin 7; DPP4: Dipeptidyl peptidase 4; KRT18: Keratin 18; SUMO2: Small ubiquitin-like modifier 2; STMN1: Stathmin1; CFHR2: Complement factor H related 2; KRT8: Keratin 8; KRT19: Keratin 19; RPL18: Ribosomal protein L18; KRT75: Keratin 75; FAM3C: Family with sequence similarity 3 member C; HIST1H2BA: Histone cluster 1 H2B family member a; CALR: Calreticulin; FAM151A: Family with sequence similarity 151 member A; SERPINA2P: Serpin family A member 2; FAM3C: Family with sequence similarity 3 member C; DAG1: Dystroglycan 1; KITLG: KIT ligand; LUM: Lumican; FABP4: Fatty acid binding protein 4; AGT: Angiotensinogen; LRG1: Leucine rich alpha-2-glycoprotein 1.

responsible for CAD should be performed with extreme caution and with careful dosing of the suspected molecules. Second, the causes of CAD may be quite different, and the aim of these studies should also take into account differentiation of the molecules or genes responsible for different aetiologies.

Non-invasive biomarkers of CAD are essentially based on proteomics and genomics.

Proteomic studies for CAD

In a review published in 2012, Bohra *et al*[11] discussed the main proteomic and metabolomic studies aimed at identifying biomarkers of CAD. Additionally, Johnston *et al*[106] reported β2 microglobulin as a urinary biomarker for CAD. In a large study by Kurian *et al*[107], 302 proteins in peripheral blood were identified as responsible for mild CAD and 509 for severe CAD, and Quintana *et al*[108] found uromodulin and kininogen in urine to be useful biomarkers for CAD. Based on a two-dimensional differential gel electrophoresis of urine, Bañon Maneus *et al*[109] found 21 proteins associated with CAD, including A1AT, α-1 β glycoprotein (A1BG), angiotensinogen (AGT), anti-TNF alpha antibody light chain, β2 microglobulin (B2M), brevin, heparan sulfate proteoglycan (HSPG), leucine-rich α 2-glycoprotein 1 (LRG1) and transferrin.

In a more recent study, Nakorchevsky *et al*[110] in a large-scale proteogenomic analysis of tissue biopsies found more than 1000 proteins associated with mild to-severe CAD.

Jahnukainen *et al*[111] in a proteomic analysis of urine in kidney transplant patients with BKVN applied surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) analysis to distinguish protein profile characteristics of BKVN but were unable to identify different proteins. More recently, Sigdel *et al*[73] found BKVN selective proteins to be associated with contractile fibers, with gene expression regulation, with glycolysis and with response to viruses. In this study the top 10 most significant urine proteins for AR, BKVN and CAN are shown (Table 8).

Recent studies on calcineurin inhibitor toxicity documented altered expression of 38 proteins *in vitro* after incubation with cyclosporine (CyA)[112], and in a clinical setting, urine N-acetylβ-D-glucosaminidase (NAG) was found to be specific for CyA-related toxicity[113].

The discovery and use of mRNAs has shed new light on CAD and on the unique form of CAD called interstitial fibrosis/tubular atrophy (IF/TA).

One recent study reported the miRNA characteristics of patients affected by IF/TA[114], in particular five miRNAs (miR142-3p, miR-32, miR204, miR-107 and miR-211) were differentially expressed in tissue biopsy samples. These miRNAs were further confirmed in the urine of patients affected by CAD. In a follow-up study by the same group[115], a selected panel of miRNAs, miR99a, miR-140-3p, mi 200b and miR-200, monitored at different time points after transplantation were found to be differentially expressed in urine according to graft outcome and useful markers in graft monitoring. In a recent study, Zununi Vahed *et al*[116] observed that urinary miRNAs exibit different behaviors in patients affected by IF/TA according to whether they received a living or cadaveric donor kidney.

In another recent study on renal biopsies of patients affected by IF/TA, miR-142-5p and miR-142-3p were significantly up-regulated, whereas miR-211 was significantly down-regulated[117]. As the same results were observed in PBCs from the same patients, the authors suggested that PBCs might be used in a non-invasive approach for monitoring kidney graft function.

Finally, evaluating miRNA profiles in transplanted patients, Iwasaki *et al*[118] found that miR-486-5p was significantly over-expressed in these patients who produced donor-specific antibodies (DSA) and exhibited biopsy-proven chronic antibody-mediated rejection (CAMR).

Genomic studies for CAD

Mas *et al*[119] used microarrays to evaluate renal tissue from patients affected by CAD with IF/TA and found up-regulation of genes related to fibrosis, extracellular matrix deposition and the immune response, as provided in Table 9. Markers of genes such as transforming growth factor beta (TGF-β), epidermal growth factor receptor (EGFR), and AGT were similarly found to be elevated in

Table 9 Genes higher (fold change higher than 6.00) expressed in renal tissue of patients affected by interstitial fibrosis/tubular atrophy[119]

Symbol	Gene name	Cytoband
IGHA1	Immunoglobulin heavy constant alpha 1	*14q32.33*
IGHG1	Immunoglobulin heavy constant gamma 1	*14q32.33*
CCR2	Chemokine C-C motif receptor 2	*3p21.31*
DFFB	DNA fragmentation factor 40 Da beta subunit	*1p36.32*
CD44	CD44 antigen	*11p13*
IFNA1	Interferon alpha 1	*9p21.3*
GZMK	Granzyme K	*5q11.2*
MMP9	Matrix metallopeptidase 9	*20q13.12*
TNFRSF17	Tumor necrosis factor receptor superfamily, member 17	*16p13.13*
CXCR4	Chemokine C-X-C motif receptor 4	*2q22.1*

urine samples.

In the multicenter CTOT-04 trial, in addition to validating the three-gene signature of CD3ε mRNA, CXCL10-mRNA and 18S rRNA, which is predictive of acute rejection, Lee *et al*[120], examined urinary mRNA by PCR and reported a 4-gene signature of mRNAs for vimentin, NKCC2, E-cadherin and 18S rRNA that was diagnostic of IF/TA.

The above-mentioned tCRM[104] is a computational gene expression score for predicting immune injury in renal allograft. A subset of 7 genes [CD6 molecule (*CD6*), inositol polyphosphate-5-phosphatase D (*INPP5D*), interferon-stimulated exonuclease hene 20 (*ISG20*), natural killer cell granule protein 7 (*NKG7*), proteasome subunit beta 9 (*PSMB9*), runt-related transcription factor 3 (*RUNX3*) and transporter 1, ATP-binding cassette subfamily B member (*TAP1*)] had higher predictive value for patients developing IF/TA over time.

A relevant international study of Genomics of Chronic Allograft Rejection (GoCAR) (Clinical Trials.gov NCT 00611702)[121] aimed to identify genes that correlate with chronic allograft dysfunction index (CADI) scores at 12 mo in patients with a normal biopsy at three months.

A set of 13 genes showed independent predictive value for the development of fibrosis (Table 10). This gene set also has a predictive value higher than that of clinical and pathological variables.

A new approach of the Mount Sinai group[122] is to utilize genomics to identify therapeutic agents for IF/TA. Based on an 85-gene signature from IF/TA molecular datasets in Gene Expression Omnibus and using a computational repurposing analysis, two new drugs, in addition to well-known azathioprine already used for AR and pulmonary fibrosis, appear to be promising: Kamferol, which attenuates TGF-β1, and Esculetin, which inhibits the Wnt/β catenin pathway. Both drugs were effective and safe in preclinical models.

BIOMARKERS TO PREDICT AND MONITOR TOLERANCE

No more than 100 cases of clinical operational tolerance (COT) have been reported in renal transplantation[123].

A number of consortia have been realized in an attempt to find valid tolerance signatures. The more important consortia are reported in Table 11[124,125].

Thirty-nine genes have been found to be up-regulated in COTs in different sites, in different patient cohorts and using different microarrays; 24 of these genes (69%) are B cell related, with CD79b and prepronociceptin (PNOC) being the more highly expressed[126-128]. Additionally, Danger *et al*[129] documented up-regulation of miR-142-3p in B cells of COT patients.

T reg cells ($CD4^+$, $CD25^+$, Fox $P3^+$) have been extensively studied in operational tolerance, though their role in COT remains unclear[128,130]. A role for natural killer (NK) cells in COTs has also been postulated[128].

In another relevant study, Roedder *et al*[131] highlighted that tolerance biomarkers are dependent on the age of the recipient and may differ according the organ transplanted and that there is a need for further validation studies. The same authors identified different biomarkers according to age and the organ transplanted.

Genomic studies for tolerance

A study on gene expression in peripheral B cells showed an up-regulation of membrane-spanning 4-domains A1 (*MS4A1*) (*CD20*), T-cell leukemia/lymphoma 1A (*TCL1A*), CD79b molecule, immunoglobulin-associated beta (*CD79B*), tolerance-associated gene 1 (*TOAG1*) and Forkhead Box P3 (*FOXP3*) genes. *TOAG1* was also up-regulated intragrafts[132].

In a recent study, a group from Northwestern University in Chicago found an important role for Treg cells. Indeed, in their study on COTs patients *vs* non-tolerant patients, the number of circulating Treg cells was significantly time-dependently higher in tolerant patients[133]. Additionally, in the same study, a role for a different 357 gene signatures of tolerance was found (Table 12).

A principal approach for identifying genes actually involved in COTs derives from comparison of tolerant patients *vs* those with immunosuppression; immunosuppressive treatment in the latter group might influence and generate bias in the gene expression signature. To overcome the problem, a multicenter study[134] reviewed a cohort of 246 kidney transplant recipients (232 with

Table 10 Thirteen genes associated with chronic allograft dysfunction identified by biopsy transcriptome expression[121]

Symbol	Gene description	Cytoband	CADI 12 mo correlation	*P* value
CHCHD10	Coiled-coil-helix-coiled- coil helix domain containing 10	*22q11.23*	0.404	2.85×10^{-5}
KLHL13	Kelch-like family member 13	*Xq23-q24*	0.369	1.49×10^{-4}
FJX1	Four jointed box 1	*11p13*	0.367	1.60×10^{-4}
MET	Met proto-oncogene	*7q31*	0.352	3.01×10^{-4}
SERINC5	Serine incorporator 5	*5q14.1*	0.318	0.0012
RNF149	Ring finger protein 149	*2q11.2*	0.28	0.0046
SPRY4	Sprouty homolog 4	*5q31.3*	0.27	0.0062
TGIF1	TGF-β induced factor homeobox 1	*18p11.3*	0.244	0.0140
KAAG1	Kidney associated antigen 1	*6p22.1*	0.24	0.0154
ST5	Suppressor of tumorigenicity 5	*11p15*	0.232	0.0197
WNT9A	Wingless-type MMTV integration site family member 9A	*1q42*	0.212	0.0332
ASB15	Ankirin repeat and SOCS box-containing 15	*7q31.31*	-263	0.0079
RXRA	Retinoid X receptor alpha	*9q34.3*	-0.3	0.0023

CADI: Chronic allograft dysfunction index.

Table 11 International research consortia in rejection/tolerance

Acronym	Description	Year
ITN	Immune tolerance network	Since 2002
IOC	Indices of tolerance	2003-2007
RISET	Reprogramming the immune system for establishment of tolerance	2005-2010
GAMBIT Study	Genetic analysis and monitoring of biomarkers of immunological tolerance	2010
The One Study	A unified approach to evaluating cellular immunotherapy in solid organ transplantation	2011
Bio-DRIM	Personalized minimization or immunosuppression after solid organ transplantation by biomarker driven stratification of patients to improve the long-term outcome and health-economic data of transplantation	2012
BIOMARGIN	Biomarkers of renal graft injuries in kidney allograft recipients	2013

GAMBIT: Genetic Analysis and Monitoring of Biomarkers of Immunological Tolerance.

immunosuppression, 14 tolerant) using the Genetic Analysis and Monitoring of Biomarkers of Immunological Tolerance method, and the investigators were able to identify a nine gene immunosuppression-independent gene signature (Table 13).

Recently, 21 genes involved in tolerance were identified at the University of California San Francisco (UCSF), in the program kidney spontaneous operational tolerance test (kSPOT). These investigators studied 348 HLA-mismatched renal transplant patients and identified 21 genes involved in COT. These 21 TOL genes were validated, and independent qPCR for the 21 genes was preformed. Additionally, the authors were able to refine and validate a three-gene assay [Kruppel-Like Factor 6 (*KLF6*), Basonuclin 2 (*BNC2*), and Cytochrome P450 Family 1 Subfamily B Member 1 (*CYP1B1*)] to detect the state of operational tolerance, with an AUC 0.95[135]. Interestingly, *BNC2* and *CYP1B1* are both related to tolerance in kidney and liver transplantation[136,137].

In conclusion, a number of studies have searched for a "tolerance signature". However, such an endeavour is difficult because of the small number of COT patients. The search for biomarkers is principally useful for identifying tolerant patients. Among the different studies, that of Newell *et al*[127], which was aimed at finding a gene expression profile for tolerant patients, and the microarray analysis of Sagoo *et al*[128] stand out in this field.

In addition, the reclassification of transplant patients according to immune risk threshold may be achieved using the cited kSORT, tCRM, uCRM and kSPOT. This might help in determining which recipients would benefit from withdrawal or minimization of immunosuppression.

FUTURE PERSPECTIVES

Several prospective research programs and clinical trials are ongoing using already-known biomarkers or are searching for new ones.

Biomarker-driven personalized immunosuppression (BIO-DrIM) is a European Consortium aimed at the Methodical and Clinical Validation of Biomarkers for guiding immunosuppression[138]. The programs of the Consortium include: (1) The targeting and partial weaning of immunosuppression in long-term liver and kidney transplant patients; and (2) biomarker analysis and data management.

The biomarker platforms of BIODrIM are as follows: (1) An ELISPOT platform for detecting donor-reactive memory/effector T cells[139]; (2) a real-time RT-PCR platform to identify molecular tolerance signatures[140]; and (3) a multiparameter flowcytometry platform to characterize circulating immune cell subsets[141].

The BIODrIM consortium is designing two clinical trials in solid organ transplantation using biomarkers for decision making.

Table 12 Immune/inflammatory molecules among the 357 gene signatures of tolerance

Categories	Diseases or functions annotation	Molecules	No. of molecules
Cell-to-cell signaling and interaction, cellular function and maintenance, hematological system development and function, inflammatory response	Phagocytosis of leukocyte cell lines	FGR, MRC1, TLR4	3
Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking, inflammatory response, tissue development	Binding of neutrophils	FGR, LSP1, TLR4	3
Antimicrobial response, inflammatory response	Antibacterial response	CARD9, FGR, LYST, NLRC4, TLR4	5
Cell-to-cell signaling and interaction, hematological system development and function, inflammatory response	Binding of professional phagocytic cells	FGR, LSP1, NOTCH2, TLR4	4
Inflammatory response	Immune response of cells	CARD9, CLEC7A, ETS2, FGR, MRC1, SCARF1, MYO7A, TLR4	8
Antimicrobial response, inflammatory response	Antimicrobial response	CARD9, CLEC7A, FGR, LYST, NLRC4, TLR4	6
Inflammatory response	Innate immune response	CARD9, CLEC7A, TLR4, TRIM59	4
Cellular function and maintenance, inflammatory response	Phagocytosis	CLEC7A, ETS2, FGR, MRC1, MYO7A, TLR4, TPCN2	7
Cell-to-cell signaling and interaction, cellular growth and proliferation, hematological system development and function, inflammatory response	Stimulation of phagocytes	IL4R, TLR4	2
Antimicrobial response, humoral immune response, inflammatory response	Antifungal response	CARD9, CLEC7A	2
Cell-to-cell signaling and interaction, cellular function and maintenance, inflammatory response	Phagocytosis of cells	CLEC7A, ETS2, FGR, MRC1,MYO7A, TLR4	6

These genes potentially predict those patients that can be successfully weaned off immunosuppression[133]. FGR: Tyrosine-protein kinase Fgr; MRC1: Mannose receptor, C type 1; TLR4: Toll-like receptor 4; FGR: Tyrosine-protein kinase Fgr; LSP1: Lymphocyte-specific protein 1; CARD9: Caspase recruitment domain family member 9; LYST: Lysosomal-trafficking regulator; NLRC4: NLR family CARD domain-containing protein 4; NOTCH2: Neurogenic locus notch homolog protein 2; CLEC7A: C-type lectin domain family 7 member A; ETS2: Protein C-ets-2; SCARF1: Scavenger receptor class F member 1; MYO7A: Unconventional myosin-VIIa; TRIM59: Tripartite motif-containing protein 59; TPCN2: Two pore calcium channel protein 2; IL4R: Interleukin 4 receptor.

Table 13 Immunosuppression-independent gene signatures predicting tolerance[134]

Symbol	Gene name	Molecular function	Biological processes
ATXN3 ↓	Ataxin 3	Ubiquitin-specific protease activity	Protein metabolism
BCLA1 ↓	BCL2-related protein A1	Receptor signaling complex scaffold activity	Apoptosis
EEF1A1 ↓	Eukaryotic translation elongation factor 1 alpha 1	Transcription regulator activity	Regulation of cell cycle
GEMIN7 ↑	Gem associated protein 9	Ribonucleoprotein	Regulation of nucleobase, nucleosides, nucleotide and nucleic acid metabolism
IGLC1 ↑	Immunoglobulin lambda constant 1	Antigen binding	Immune response
MS4A4A ↑	Membrane-spanning 4-domains, subfamily A, member 4A	- - -	- - -
NFκBIA ↑	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	Transcription regulator activity	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
RAB40C ↑	RAB40C, member of RAS oncogene family	GTPase activity	Cell communication, signal transduction
TNFAIP3 ↓	Tumor necrosis factor, alpha-induced protein 3	Transcription regulator activity	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism

↓Immunosuppression-free gene expression downregulated in tolerant patients; ↑Immunosuppression-free gene expression upregulated in tolerant patients; BCL2: B-cell lymphoma 2.

The trial LIST[138] will apply molecular signatures to guide immunosuppression in liver transplant patients.

The kidney transplant trial design of BIODrIM is Cellimin, a prospective multicenter randomized trial utilizing IFNγ ELISPOT to stratify kidney transplant recipients into high/low responders. Only low-responder patients will be randomized to receive either standard immunosuppression or low-dose immunosuppression. The trial will evaluate the donor specific cellular alloresponse for immunosuppression minimization (EudraCT-Number: 2013-005041-37)[142].

Another European research program is "Biomarkers of Renal Graft Injuries in kidney allograft recipients" (BIOMARGIN)[143], which has the aims to: (1) select and validate blood or urine biomarkers at different-omics levels related to allograft lesions; and (2) select

and validate biomarkers as early predictors of CAD. The research will allow for selecting the best candidate biomarkers and biomarker signatures. In addition, the work will evaluate the sensitivity, selectivity, false positive value and false negative value of biomarkers. Finally, one goal of the study is to select biomarker signature predictors of three-year graft outcomes.

By using the aforementioned biomarkers of kSORT, the TITRATE trial has the aim of testing immunosuppression Threshold in Renal Allografts to improve the estimated glomerular filtration rate (eGFR). Overall, the main outcomes of the trial are the rate and severity of acute rejection and the CADI score at one year based on protocol biopsy. Evaluation of eGFR is also a principal endpoint. The study is ongoing in Mexico and at UCSF[144].

Another Clinical Trial, NIH UO1 trial TASK, employs the biomarkers of kSORT, uCRM, and tCRM. The TASK trial has the aim of evaluating Treg adoptive therapy for subclinical inflammation in kidney transplantation by comparing the results of three patients' cohorts according to surrogate markers of the immune response[145].

The Precision Medicine Offers Belatacept Monotherapy study[146] is being conducted at four centers in the United States, Spain, France and Mexico. The trial has the aim of determining the safety and feasibility of converting kidney transplant recipients to Belatacept monotherapy. In addition, the trial has the goal of evaluating the percentage of patients who can be converted to a Belatacept regimen of once every 8 wk. The patients enrolled in the trial will have a quiescent immunologic profile evaluated by kSORT, uCRM and tCRM. Only those with elevated kSPOT will be tested for the once every 8-wk administration.

The epithelial-to-mesenchymal transition (EMT) is a process in which fibrosis is generated due to the transformation from the epithelial to mesenchymal phenotype. The process is induced and facilitated by several molecular signatures, among which TGF beta, EGF, insulin like growth factor 2 and fibroblast growth factor 2 (FGF2) are prominent[147]. An interesting ongoing trial is Prediction of Chronic Allograft Nephropathy (Prefigur)[148]. By using non-invasive biomarkers and evaluating urinary cells in the first year post-transplantation, the investigators are developing a non-invasive approach for predicting fibrosis as a substitute of allograft biopsy, *via* longitudinal assessment of the mRNA expression level of genes implicated in EMT fibrogenesis.

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REVIEW

Historical perspective of cell transplantation in Parkinson's disease

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Abstract

Cell grafting has been considered a therapeutic approach for Parkinson's disease (PD) since the 1980s. The classical motor symptoms of PD are caused by the loss of dopaminergic neurons in the substantia nigra pars compacta, leading to a decrement in dopamine release in the striatum. Consequently, the therapy of cell-transplantation for PD consists in grafting dopamine-producing cells directly into the brain to reestablish dopamine levels. Different cell sources have been shown to induce functional benefits on both animal models of PD and human patients. However, the observed motor improvements are highly variable between individual subjects, and the sources of this variability are not fully understood. The purpose of this review is to provide a general overview of the pioneering studies done in animal models of PD that established the basis for the first clinical trials in humans, and compare these with the latest findings to identify the most relevant aspects that remain unanswered to date. The main focus of the discussions presented here will be on the mechanisms associated with the survival and functionality of the transplants. These include the role of the dopamine released by the grafts and the capacity of the grafted cells to extend fibers and to integrate into the motor circuit. The complete understanding of these aspects will require extensive research on basic aspects of molecular and cellular physiology, together with neuronal network function, in order to uncover the real potential of cell grafting for treating PD.

Key words: Parkinson's disease; Cell replacement; Animal models; Nigrostriatal pathway; Striatum; Dopaminergic loss

Core tip: The first studies on cell transplantation for

Parkinson's disease were published during the early 80s. Since then, it has been shown that different cell types induce functional benefits but with high variability among subjects. Here, we first provide a general overview of the field during its early years. Then, we discuss some factors associated with the functionality of the graft based on the latest findings, and highlight the importance of understanding basic aspects (*e.g.*, factors influencing graft integration) which ultimately could contribute to reducing the variability of the functional outcome-an important requirement for its application in the clinic.

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INTRODUCTION

The transplantation of different tissues into the brain began as an experimental approach for understanding fundamental aspects of the development and function of the central nervous system. The first transplant in an animal model of Parkinson's disease (PD) was performed in 1979 with the objective of determining whether grafted dopamine-producing cells were able to reduce the motor alterations in the animal model[1,2]. These and other initial reports of graft tissue survival in the brain, and its beneficial effects on a PD animal model, contributed to the beginning of cell grafting era in PD, including both basic and clinical research approaches. Nearly 40 years after the first studies in this field, there is continuing interest in the development of cell-replacement therapies for treating PD, with a particular focus on the search for optimal cell-sources for grafting. The objective of this review is to perform a general description and a critical evaluation of our current understanding of the mechanisms underlying the success of cell-replacement therapy in animal models of PD. We will mainly focus on the mechanisms underlying the functionality of the grafts when evaluated using pharmacological tests, and on the comparison of the results obtained principally with fetal ventral mesencephalic cells (FVM) and embryonic stem cells (ESCs)-derived midbrain dopaminergic neurons. Ultimately, the purpose of this review is to provide a perspective of what has been gained relative to the prevailing knowledge during the starting point of this research area: Basically, that in order to provide a long-term benefit in PD motor symptoms, functional integration of the transplanted cells into the host brain circuit is essential.

EARLY YEARS OF CELL GRAFTING INTO THE BRAIN

The earliest known report of neural tissue transplantation into the brain was conducted by Thompson[3] in 1890. He published a brief description of the transient survival of grafted cat cortical tissue into the brain of a dog, in a work entitled "Successful brain grafting"[3]. In 1907, in another attempt to prove that brain grafting was possible, Del Conte[4] implanted fetal cortex tissue into an adult mammalian brain, showing similar results to those reported by Thompson. In 1909 Ranson provided evidence that suggested that postnatal nervous tissue, the cervical ganglion obtained from 1-wk-old rats, survived when grafted into the adult cortex[5]. Later, in 1917 Dunn found that rat neonatal cerebral cortex tissue transplanted into the adult rat brain was able to survive, grow, and even exhibited myelinated fibers[6]. Other studies were performed during the following years (*e.g.*, Ref[7,8]), which together with those described thus far, constitute the earliest antecedents for cell transplantation.

The functional consequences of brain transplants were not evaluated until 1979[1,2] using the 6-hydroxydopamine (6-OHDA) animal model of PD, which was developed 10 years before[9]. This model allows the selective destruction of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of only one hemisphere of a rat's brain[9]. The motor asymmetry observed in this toxin-based model is characterized by a turning behavior contralateral or ipsilateral to the side of the lesion, and is induced by the systemic administration of dopaminergic agonists (amphetamine or apomorphine) (Figure 1A and B)[9,10]. These experimental approaches allowed to test the functional consequences of cell transplantation by grafting dopamine-producing cells[1,2,11]. The general assumption was that, since motor asymmetry is a consequence of a decrement in dopamine in the striatum, then that asymmetry could be reversed by grafting dopaminergic cells, as long as they release dopamine in the host (Figure 1C and D).

CELL GRAFTING IN PD: THE PIONEERING STUDIES (1979-1990)

FVM grafts in pre-clinical studies

Cells derived from FVM tissue were the first type of cells used for brain grafting in the 6-OHDA rat model of PD[1,2] (for a timeline of pre-clinical studies see Figure 2). This tissue was selected because it contains dopaminergic neurons[12]. In 1979 and 1980, two independent studies confirmed that FVM cells were able to survive (from few to approximately 4000 surviving grafted cells observed 1-7 mo after transplantation), to extend projections into the host striatum after being grafted into the lateral ventricle (Figure 3)[1] or in a cavity at the surface of the striatum (Figure 3)[2], and to reduced circling behavior induced either by apomorphine[1] or amphetamine[2] by approximately 50%, when compared to measurements of motor asymmetry before transplantation. These results were encouraging as they were the first demonstration of a functional outcome induced by grafting exogenous cells in the brain.

The mechanism underlying the functional effects of the grafts was proposed to be the dopamine released

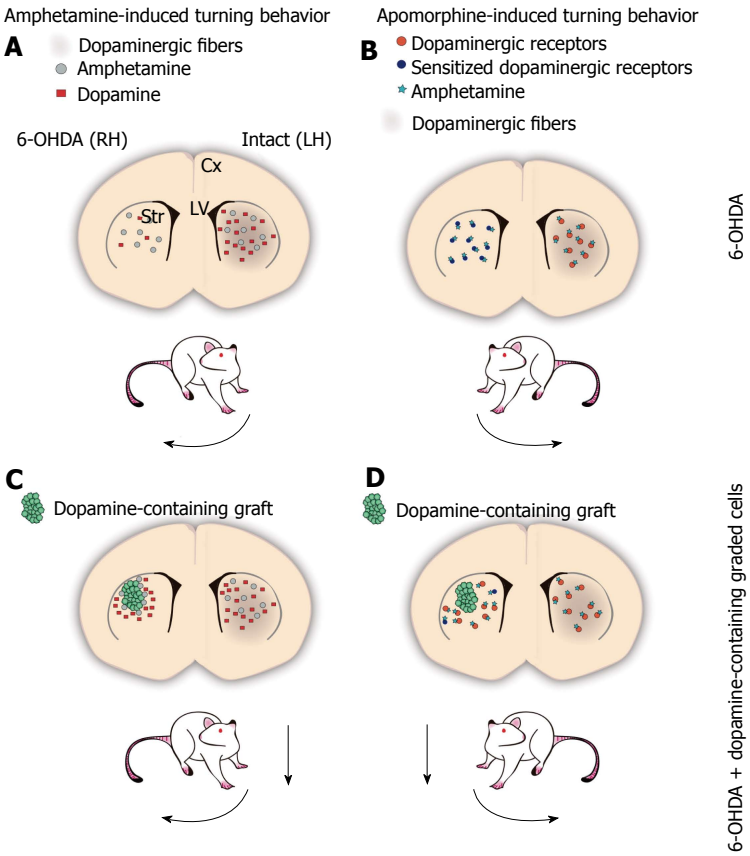

Figure 1 The 6-OHDA rat model of Parkinson's disease. A-D: Schemes of a coronal representation of the rat brain. Dopaminergic fibers are depicted with brown shadowing, which is lacking in the 6-OHDA-lesioned hemisphere; A: Amphetamine (grey circles) administration promotes the release of dopamine (red squares) from the intact dopaminergic terminals of the striatum, disproportionally increasing dopamine concentration in the non-lesioned side relative to the lesioned side, as the latter contains fewer (or none at all) dopaminergic terminals. The asymmetry in extracellular dopamine levels between both hemispheres induces the stereotypical behavior known as circling or turning behavior, ipsilateral to the lesioned side (curved arrow next to the rat); B: Apomorphine is a dopaminergic receptor agonist that can activate postsynaptic dopamine receptors in the striatum (orange circles). 6-OHDA-induced dopaminergic denervation in one hemisphere of the striatum, results in postsynaptic supersensitivity to dopamine in the lesioned side (sensitized dopamine receptors are represented as dark blue circles), such that apomorphine (teal stars) stimulation increases the activity in the lesioned side to a greater extent than in the non-lesioned side. The supersensitivity effect promotes that lesioned animals turn contralateral to the lesioned side after apomorphine administration (curved arrow); C: Amphetamine stimulates dopamine-containing cells (green circles) grafted into the denervated striatum increasing extracellular dopamine concentration in the lesioned side, which leads to a decrement in motor asymmetry (dashed arrow); D: Grafted cells that release dopamine decrease the supersensitivity effect on the lesioned hemisphere, normalizing the response to dopamine or agonists relative to the non-lesioned side. Thus, after apomorphine administration, grafted animals decrease their turn number (dashed arrow). Cx: Cortex; LH: Left hemisphere; LV: Lateral ventricles; RH: Right hemisphere; Str: Striatum.

from FVM cells (Figure 1C and D). However, the first studies found that some animals with surviving grafts did not exhibit any improvement in turning behavior. Several authors using either the same cell type[13-19] or a different cell source[11,20] have replicated these observations, which has not received a complete explanation to date. However, by that time, Björklund and Stenevi[2] proposed that fiber ingrowth from grafted cells into the striatum was, together with the release of dopamine, the determining factors for producing a reduction in circling behavior. Subsequently, a correlation between the reduction in amphetamine-induced turning behavior and the degree of fiber ingrowth was reported[21]. The observations on the variability in the motor improvement in grafted animals with surviving transplants was also found to correlate with the degree of dopaminergic lesion[1,22] and graft survival[23].

One year after the first reports of cell grafting in an animal model of PD, evidence confirmed that dopamine was present in the lesioned striatum of FVM grafted animals[14]. Dopamine tissue-content was found to correlate with the reduction of circling behavior induced by amphetamine. It was also found that a restoration of at least 3% of normal dopamine levels in the striatum was sufficient to reduce the motor asymmetry[24]. However, these observations only demonstrated that mesencephalic transplants contain the neurotransmitter, but not that they release it. In 1983, Freed *et al*[25] provided more direct evidence for the role of dopamine on motor improvement in the 6-OHDA model of PD. The authors suggested that the graft can release dopamine spontaneously on a tonic basis, reversing the supersensitivity effect caused by dopaminergic denervation by directly quantifying the binding of dopamine to its receptors using a dopamine-receptor binding assay. A few years later, Zetterström *et al*[26], conducted a study using an *in vivo* dialysis assay, where they corroborated that mesencephalic transplants release dopamine spontaneously, and after amphetamine administration. One-year later, the same group observed that dopamine release was higher in animals with more surviving grafted cells and more fiber ingrowth, reaching about 85% of normal dopamine levels under basal conditions[23].

In addition to the reduction in turn number induced either by amphetamine or apomorphine, FVM grafts were shown to reduce some aspects of spontaneous abnormal behaviors observed in the PD animal model, such as sensorimotor orientation deficits and asymmetric limb use[15,27]. However, other studies failed to replicate these results[28,29].

Nowadays, FVM-derived cells remain as one of the most promising sources for cell grafting[30], and much more information has been obtained by using this cell source compared with other cell types. However, a major problem related to the use of FVM tissue as a source for cell grafting was the ethical concern due to the use of fetal-derived tissue, which led to the search for alternative cell-sources.

Adrenal medulla grafts in pre-clinical studies: Dopamine vs neurotrophic effects

Chromaffin cells are neuroendocrine cells that synthesize and release catecholamines from the adrenal medulla (AM) into the bloodstream in response to sympathetic

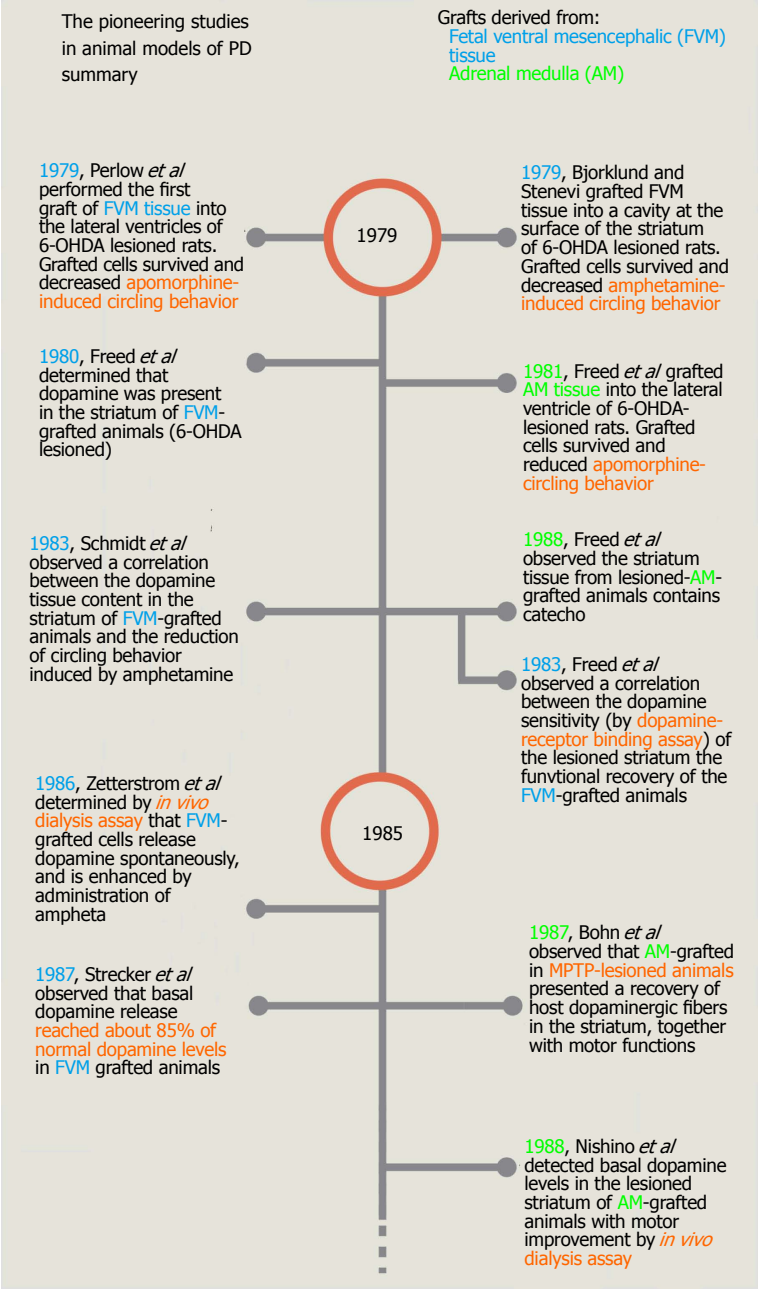

Figure 2 Timeline of the pioneering studies on cell transplantation in animal models of Parkinson's disease. This timeline shows only a few of the studies performed during the first 10 years of cell grafting in animal models of PD. Most of these studies were selected because they were the first published reports of either the use of a new animal model of PD, a site of grafting, a type of cell or a specific technique. PD: Parkinson's disease; FVM: Fetal ventral mesencephalic cells; AM: Adrenal medulla.

stimulation, triggering the fight-or-flight response. This cell source was chosen for use in cell replacement therapy mainly due to the capacity of chromaffin cells to produce dopamine (for review[31]). The first published report using AM tissue grafted in a PD animal model was conducted by Freed *et al*[11]. They demonstrated that AM grafted into the lateral ventricle of 6-OHDA-lesioned rats reduced apomorphine-circling behavior by 20%-50% relative to the initial values before grafting, and this effect lasted for at least 2 mo[11]. However, the cells extended only very few fibers into the host tissue and the mean number of surviving cells was approximately 1535 two months post-grafting[11]. In addition, the animal with the highest number of surviving grafted cells (approximately 4000) did not reduce its circling behavior.

During the eighties it was assumed that the mechanism of action of AM grafts was similar to FVM cells, consisting of the diffusion of high concentrations of dopamine spontaneously released by the graft[11]. Later, it was demonstrated that AM grafts contain high concentrations of adrenaline and noradrenaline, but low concentrations of dopamine[32], mirroring their native characteristics in the AM. When the release of these catecholamines by AM grafts was evaluated by *in vivo* dialysis assays, the authors of the study detected basal dopamine levels only in those animals with motor improvement. Surprisingly, the dopamine levels found were only 50% lower than normal values in the non-lesioned striatum, despite the low survival of grafted chromaffin cells (approximately 50-600 cells)[20]. However, other authors found that the results obtained from chromaffin cell grafts were highly variable and unpredictable in terms of survival and functional outcome, especially when grafts were placed into the striatum (intraparenchymal)[33].

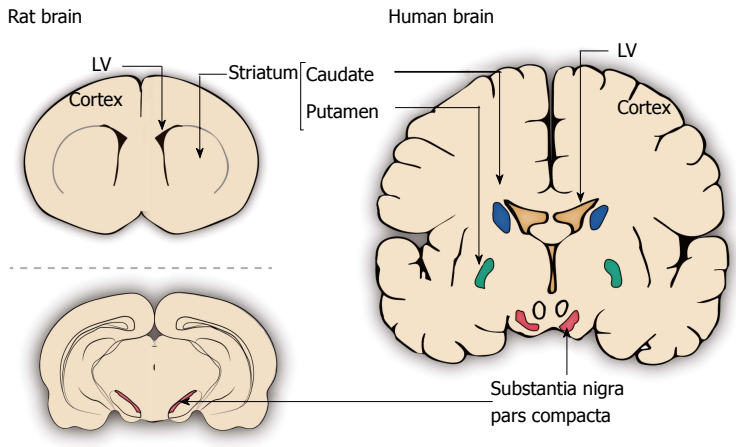

Figure 3 Schematic representation of different sites in the rat and human brains used for grafting in Parkinson's disease. The depicted grafting sites include the lateral ventricles (LV), the striatum (in rat) or caudate nucleus and putamen (in human) and the substantia nigra pars compacta. The above schemes are coronal sections of the rat striatum and human caudate (blue) and putamen (green) together with the substantia nigra pars compacta (red). The scheme below is a coronal section at the level of the rat substantia nigra pars compacta (red).

The discrepancies observed when AM-tissue was grafted in the 6-OHDA model of PD, together with results derived using a different model of PD, the 1-methyl-1,2,3,6-tetrahydropiridine (MPTP)[34], led the scientific community to suggest a different mechanism of action for chromaffin cell grafts: A neurotrophic effect. In this regard, different authors observed that MPTP-lesioned animals with chromaffin cell grafts presented an enhanced recovery of host dopaminergic fibers in the grafted striatum of mouse[35] and monkeys[36], together with a transient functional recovery[37]. These studies suggested a neuroprotective action of the chromaffin cells, which induced the reappearance of tyrosine hydroxylase (TH) immunoreactivity (THir) or the sprouting of surviving host fibers, leading to an increment of dopamine released by the endogenous cell (for review[31]). However, a direct comparison of AM grafts to FVM-derived cells in 6-OHDA lesioned rats demonstrated that AM grafts were less effective in terms of functionality and in their long-term survival, even when AM grafts were placed in the lateral ventricles[38], a site which was assumed to induce a better survival of AM grafts. Thus, despite some studies showing transitory and modest recovery of motor function, AM-derived cells were shown to induce variable and unpredictable results, probably derived from their different mechanism of action compared to FVM grafts.

Clinical studies: A brief description

Although this review is focused in studies using animal models of PD, it is also important to provide at least a general overview of the clinical trials that have been done using both cell sources described above (FVM- and AM-derived cells) (for a timeline of the pioneering studies see Figure 4). There are several extensive reviews aimed at describing critically and in a deeper way the results derived from clinical studies (*e.g.*, Ref[30]).

AM-derived cells were the first to be tested in human patients with PD, with similar results as those observed in animals: Variable and transitory restoration of some motor function[39-41]. Autologous chromaffin cells were first grafted in three different places: The caudate nucleus (Figure 3)[39], the putamen (Figure 3)[40], or in a cavity made at the interface between the caudate nucleus and the lateral ventricles (Figure 3)[41]. In the two first studies the patients showed only moderate recovery that did not last longer than a few months[39,40]. However, by placing the grafts in proximity to the lateral ventricles, other authors reported that one of their two patients showed motor improvements that persisted for at least 10 mo after the grafting procedure[41]. As a result, many clinical studies were done worldwide (*e.g.*, Ref[42-46]) despite the fact that the original articles only reported transitory and modest improvements. As described in a comprehensive review on the topic by Barker *et al*[30], the scientific community started to be concerned about the clinical trials that were taking place, due to the poor or absent functional outcome induced by the AM grafts, the frequent complications from the surgery (*e.g.*, psychiatric alterations), and the fact that post-mortem studies revealed a poor survival of the grafted cells. This led to the abandonment of the use of AM tissue for transplantation.

FVM tissue was the second cell source to be grafted in patients with PD. The grafted tissue was placed into the caudate nucleus[47], the putamen (*e.g.*, Ref[48,49]), both sites (*e.g.*, Ref[50]), in a cavity made at the interface between the caudate nucleus and the lateral ventricles[51] and even directly into the SNpc (Figure 3)[52]. Unfortunately, the results varied from clear benefits to poor or none, but there were also promising results showing improvements by [^{18}F]-DOPA uptake by positron emission tomography (PET) imaging[47].

One of the most controversial issues with these studies was the lack of control groups to discard a placebo effect. In 2001, Freed *et al*[53] performed the first double-blind study that included a placebo control group, in which some patients received FVM cell-grafts bilaterally implanted in the putamen, and observed a modest recovery compared with the sham group. Other double-blind studies were done during subsequent years with a similarly variable symptomatic outcome[54]. Another important issue that became apparent several years after the surgery was that some of the grafted patients started to develop dyskinesias (involuntary movements) as a side effect of the transplant (see[55] for review).

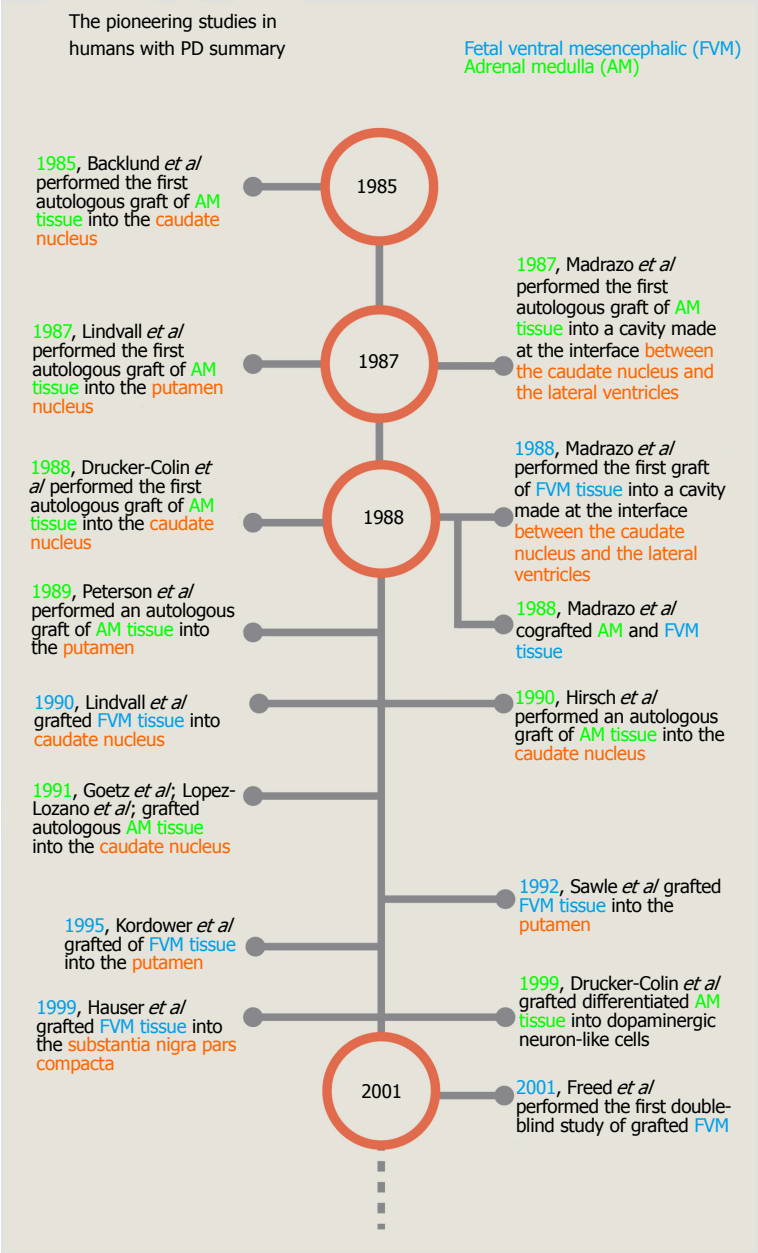

Figure 4 Timeline of the pioneering studies on cell transplantation in human patients with Parkinson's disease. This timeline shows only a few of the studies performed during the first 15 years of cell grafting in patients with PD. Most of them are the first published reports in which, a new site of grafting or a new type of cell were used. PD: Parkinson's disease.

Many clinical studies were subsequently done using FVM cells, chromaffin cells or other types of cell sources including retinal pigmented epithelial cells attached to microcarriers[56,57], adult neural stem cells[58] and autologous bone marrow-derived mesenchymal stem cells[59], all with similar results: Some patients showed moderate recovery, whereas others showed poor or no recovery at all (for review see[60,61], visit http://clinicaltrials.gov for clinical NIH-funded trials currently underway, Table 1). The highly variable results obtained even to date strongly argue that some of the key requirements for this type of therapeutic option to work are still unknown.

WHAT DO WE KNOW NOW?

The study of graft-associated mechanisms producing motor improvements in animal models of PD has been largely done using experimental paradigms with a strong bias towards the role of dopamine. However, actually we know that several additional factors also somehow influence the functional motor recovery. These include the degree of survival of the graft, the capacity of the graft to extend fibers into the host, the ability of these fibers to establish functional connections with host cells and the extrinsic factors that influence all the previously mentioned aspects. The next section of this review focuses on comparing the facts that we knew in the early years with the latest advances in the field. We will describe the results derived using two cell types, which have been widely demonstrated to possess the greatest capacity to survive and to decrease the circling behavior and improve other motor functions in animal models of PD: FVM-derived cells and ESC-derived dopaminergic neurons.

Table 1 Current clinical trials (2013-2016)

	Type of cells	Site of procedure	Age of patients	No. of patients	Control group(s)	Phase[2]	Current status and notes
The University of Texas Health Science Center, United States. NCT02611167[1]	Allogeneic bone marrow-derived mesenchymal stem cell	Delivered intravenously	45-70	20	No	I and II	Nov 2017. Starts on May 2016
ISCO-Florey. Cyto Therapeutics Pty Limited. Australia. NCT02452723[1]	Human parthenogenetic stem cells-derived neural stem cells	Striatum and SNpc	30-70	12	No	I and II	Approved from the TGA of Australia (received on December 2015)
University of Saskatchewan and Manitoba, Canada. NCT02538315[1]	Fetal dopaminergic grafts	NS	18 and older	30	NS	NS	Study type: Observational. Using [^{18}F]FDOPA PET/CT to monitor the effectiveness of grafts. Started on December 2015
University of Kentucky, United States. NCT01833364[1]	Autologous peripheral nerve	SNpc	40-75	16	No	NS	Started on 2015.
TRANSEURO, Europe. NCT01898390[a]	FVM Tissue	NS	30-68	40	Yes (no surgery)	I	No updates. Patients undergoing deep brain stimulation surgery Enrolling participants.
CHA University, South Korea. NCT01860794[1]	Mesencephalic neural precursor cells	NS	18-70	15	NS	I and II	No updates since December 2014 Started on 2013. No updates
Living cell technologies. Auckland City Hospital, New Zealand. NCT01734733[a]	NTCELL [immunoprotected (alginate-encapsulated) choroid plexus cells]	NS	40-70	NS	NS	I and II	Started on 2013. No updates

[1]Is the ClinicalTrials.gov identifier. For more information and other trials visit the website; [2]Clinical phases: I: Test a new treatment in a small group to evaluate its safety, dosage range and side effects; II: Treatment in a small group to see its effectiveness and to further, evaluate its safety. NS: No specified; TGA: Therapeutic Goods Administration.

Graft survival and the effects of grafting into the striatum and the SNpc

The survival of the grafted cells is modified by different factors including the age of the donor tissue, the graft composition and the location of the graft.

The relation between survival and functional recovery has been studied by different authors[17,19,23,62,63]. FVM tissue is usually obtained from 12.5-d-old mouse embryos or 14-d-old rat embryos. However, it has been observed that the survival of intra-striatal grafts of FVM-derived dopaminergic neurons is higher when 12-d-old rat embryos are used[64]. Interestingly, the increment in survival of grafted FVM cells (derived from rat embryos of 12 d *vs* 14 d) is not necessarily accompanied by an equivalent improvement in the functional outcome. This suggests that a critical number of cells is required for improvement, above which a higher survival does not contribute to further improvement[65]. Sauer *et al*[19] estimated that approximately 2000 surviving cells were necessary for complete recovery of turning behavior, whereas only 600 cells were necessary for a moderate level of recovery. It is important to note that, in that study, the improvement observed in four animals with 600-1500 surviving cells ranged from negligible to low[19]. In other reports, it has been observed that an even smaller number (100-200) of surviving TH^+ cells was sufficient to obtain a 50% reduction in turning behavior (*e.g.*, Ref[17,62]). More recently, using human FVM cells, it was observed that at least 657 TH^+ surviving cells were necessary to induce a significant reduction (50% relative to the initial circling behavior before grafting) in apomorphine- induced circling behavior[66]. Similarly, using human ESC-derived midbrain dopaminergic neurons, a complete recovery of amphetamine-induced circling behavior was achieved with approximately 986 TH^+ surviving cells[67]. Therefore, in general, the studies that have correlated survival of the grafted cells with behavioral improvement have, surprisingly, found that a very small number of cells are sufficient to produce a robust motor improvement.

An additional factor to be considered in the case of FVM-derived cells is that the age of the donor tissue in turn influences the composition of the grafted cells. The developing mesencephalon contains two major sub-populations of neurons: A9 and A10 neurons[12,68]. The A9 sub-population in particular corresponds to dopaminergic neurons that will form the SNpc, whereas the A10 neurons are dopaminergic neurons that form the ventral tegmental area. Each subtype differs in multiple characteristics, including their morphology, their protein-expression profile and their target areas in the brain (SNpc in the dorsal striatum and ventral tegmental area in the ventral striatum). Since FVM grafts contain a mix of these two sub-populations[69,70], researchers started to elucidate the role of each subtype on the functional outcome induced by the graft. A9 neurons were found to be critically important for a major functional recovery, due to these grafted-cells

innervating the regions of the striatum corresponding to the areas normally innervated by dopaminergic neurons from the SNpc[71].

Thus far, we have only discussed ectopic sites (*i.e.*, located outside the SNpc) for grafting as a therapeutic approach to reverse the motor alterations observed in PD. However, we have to consider that dopaminergic cells from the nigrostriatal pathway are part of a complex circuit that receives regulatory inputs from other structures (*e.g.*, SN pars reticulata). In agreement with this, it has been observed that intrastriatal grafts do not ameliorate all the symptoms associated with degeneration of the nigrostriatal pathway, since the proper function of the basal ganglia circuitry is far from being restored[16,28,65,72]. Current approaches on this front focus on the possibility of reconstructing the nigrostriatal pathway, by grafting cells into the SNpc (Figure 3) and directing their fibers to reestablish the lost dopaminergic circuitry in the striatum[73]. The first studies that attempted this procedure succeeded in demonstrating that FVM grafts survive when placed into the SNpc and that, in some cases, the neurons extended projections into the striatum and induced some reduction in drug-induced circling behavior[74-79]. However, the survival of FVM cells grafted into the SNpc was less prominent as compared to intra-striatal grafts[74,78,80].

Fiber ingrowth and dopamine release

The occurrence of fiber ingrowth from the graft into the host depends in part on the type of cell used. Intra-striatal grafts of FVM cells[67], ESC-derived dopaminergic neurons[67] and induced pluripotent stem cells (iPSC)-derived dopaminergic neurons[81] have been shown to extend fibers into the host striatum. It has been suggested that the extension of projections is important for mesencephalic grafts[13,16,21,23,27], although FVM-grafts have been shown to produce motor improvement without any detectable projections[1,82]. However, it is reasonable to consider that the greater the extension of the graft projections, the further the molecules they release can diffuse. In addition, with more and longer projections, the establishment of synaptic contacts between the host cells and the graft becomes more likely.

Certainly, an ideal scenario for intra-striatal grafts is one in which dopamine release and clearance are regulated by the necessities of the host circuit. Different authors have shown that FVM grafts release dopamine under basal conditions, and that the release can be enhanced by stimulation with amphetamine[18,26] or high extracellular potassium[83,84]. This has also been demonstrated for ESC-derived dopaminergic neurons[67,85]. Notably, these two types of cells have been shown to deliver sufficient dopamine into the striatum to restore its concentration to normal levels[67,85]. Interestingly, a recent study showed that grafts of FVM cells placed into the SNpc increased striatal dopamine levels to 77% compared to lesioned animals[86]. This study also observed extensive axonal growth from the grafted cells (confirmed by grafting cells from transgenic mice overexpressing green fluorescent protein, GFP) that reached the striatum, together with a significant behavioral recovery in the apomorphine-induced rotation of 94% relative to the initial rotation numbers before grafting[86]. Another study published the same year showed similar results[87], and demonstrated that over-expression of glial cell-derived neurotrophic factor (GDNF) enhanced survival and axonal growth from the grafted cells positioned in the SNpc. The authors also observed a reduction in turn number induced by amphetamine of approximately 75% relative to the initial values before grafting in GDNF-treated animals, which lasted for at least 12 wk[87]. In a more recent study, Grealish *et al*[67] demonstrated that human ESC-derived dopaminergic neurons (A9 and A10 phenotypes) can restore dopaminergic transmission in the transplanted striatum, as occupancy of D2/D3 receptors by dopamine measured using PET showed dopamine binding levels that were similar to the non-lesioned side. More importantly, the study demonstrated that human ESC-derived midbrain dopaminergic neurons grafted into the SNpc provided widespread innervation that extended more than 10 mm throughout the forebrain, with dense innervation in the striatum (A9 subtype), as well as nucleus accumbens, amygdala and frontal cortex (A10 subtype), which are normally innervated by endogenous dopaminergic fibers from the SNpc. In addition, they obtained similar results using human FVM, with an average axonal number of 2169 for the FVM cells and 2453 for the human ESC-derived cells[67]; although, the functional effects of the nigral grafts were not determined in this study. Taken together, these findings are encouraging, suggesting that the reconstruction of the dopaminergic pathway is a plausible approach. However, more research is necessary, to determine whether normal connectivity and physiology are established by the grafted cells into the SNpc. In this regard, it seems that the projections extended by the grafted cells are highly specific, as they connect exclusively to targets that are normally innervated by dopaminergic fibers from the SNpc (for a review on this topic see[73]).

Establishment of connections

A property of central importance for the grafted cells is their capacity to integrate into the host circuit by establishing functional synaptic connections with other cells. This feature marks a difference between grafted cells that function only as release-pumps for dopamine and trophic factors, and those that integrate into the circuit and respond to the physiological needs of the site.

Different sources of evidence support the idea that some types of grafted cells, especially FVM cells and human ESC derived-dopaminergic neurons, establish synapsis with the host cells[88-93]. Electrophysiological studies were initially difficult to perform, as no direct method existed for differentiating the graft from the host cells. Hence, in early electrophysiological studies the recorded cells were chosen blindly, and later identified by THir or by their electrophysiological properties[88,89].

These electrophysiological recordings showed that host striatal cells close to THir fiber projections of FVM cells decreased their firing rates to levels normally observed in a healthy striatum[89]. In contrast, cells located far from the graft or graft-projections presented altered firing rates[89]. Additionally, Freund *et al*[90] demonstrated by using electronic microscopy that FVM cell grafts establish synapses with the dendritic shafts and spines of the striatal neurons, including medium spiny neurons and giant cholinergic interneurons. However, they failed to track reciprocal afferent connections to the graft from the host striatum[90]. Evidence of synaptic connections, both from graft to host and from host to graft, was later observed by other authors using immunostaining for postsynaptic and presynaptic markers and electron microscopy[91]. These results confirmed that some FVM cell grafts have the capacity to integrate into the host circuit and induce changes in host cell firing rates. Concurrently, to identify electrically active afferent and efferent connections of the graft to the host cells, Arbuthnott *et al*[88] grafted FVM cells in the striatum and implanted stimulating electrodes under the grafts in the striatum but also in the frontal cortex, locus coeruleus or dorsal raphe nuclei of 6-OHDA-lesioned animals. They found that grafted cells fired action potentials after striatal stimulation in a similar manner as naïve SNpc dopaminergic neurons, but remarkably, only in those animals in which rotational behavior was compensated and had longer antidromic latencies[88]. They also observed that some grafted cells were activated after stimulation in the frontal cortex, locus coeruleus or raphe nuclei[88].

More direct evidence supporting electrical activity and connectivity of grafts has been recently obtained using FVM grafts derived from transgenic mice expressing GFP under the control of the TH gene promoter, and measuring their electrical activity with whole-cell patch clamp recordings[92]. They observed that a higher proportion of grafted cells in the lesioned striatum fired spontaneous action potentials than grafted cells in the non-lesioned striatum. However, the firing frequency was similar for both[92]. Furthermore, they measured lower frequency of inhibitory and excitatory postsynaptic currents in cells grafted into lesioned, as compared to non-lesioned, animals[92]. Based on these data, the authors suggested that dopamine levels in the striatum could modulate the activity of grafted cells by the activation of D_2 autoreceptors in FVM cells. Another possibility is that the grafts in non-lesioned animals received more GABAergic synaptic inputs[92].

The evidence presented thus far did not confirm that dopamine release was regulated by electrical activity, and that the release was responsible for the functional recovery observed in behavioral experiments. Interestingly, Dell'Anno *et al*[94] were able to control the electrical properties and neurotransmitter release of grafted reprogramed dopaminergic neurons by using designer receptors exclusively activated by designer drug technology. The authors demonstrated that the functional outcome is higher when the neural activity of the striatal-grafted cells is stimulated by clozapine-N-oxide (the pharmacologically inert molecule that activates the designed receptor expressed by the cells), achieving similar results to those observed using FVM tissue[94]. *In vitro*, stimulation of the reprogramed cells resulted also in an increment in neural activity (number of spikes per second) together with an increment of dopamine release[94].

Using a different approach to control the neuronal activity of the grafted cells in order to understand its relation to the functional outcome, Steinbeck *et al*[93] grafted differentiated mesencephalic dopaminergic neurons derived from human ESC that expressed the inhibitory light-activated chloride pump halorhodopsin (eNpHR3.0-EYEP, also known as HALO). After corroborating the functionality of the cells *in vitro*, they were grafted into the striatum of 6-OHDA lesioned immunodeficient mice. The authors observed that transplanted animals gradually decreased their amphetamine-induced turning behavior for a period of 4 mo[93]. Electrophysiological recordings on brain slices showed that the grafted cells produced action potentials that ceased after illumination (*i.e.*, activation of the HALO-mediated chloride conductance). It was also corroborated that grafted cells are able to modulate the activity of spiny medium neurons, and that they receive functional glutamatergic inputs from the host cells[93]. *In vivo* studies performed in freely moving grafted animals showed that the reduction of spontaneous rotations and sensorimotor deficits evaluated with the corridor test is dependent on graft activity, as optogenetic silencing of the cells reversed the recovery[93]. To test the dependence of recovery on dopamine release by grafted cells, the animals were injected with apomorphine before optogenetic silencing. The authors observed that after illumination the recovery of the behavior was still present, as host dopaminergic receptors were expected to be occupied by apomorphine. This study provides an appropriate strategy to interrogate the mechanisms underlying the functionality of grafted cells. In general, grafted cells have been proven to be able to integrate into the host tissue but more experiments are necessary for a complete understanding of their role in the population dynamics of the striatal circuit.

GENERAL DISCUSSION: LOOKING INTO THE FUTURE, BACK TO BASICS

After the studies by Perlow[1] and Björklund and Stenevi[2], several authors have replicated their results with the same type of cells as well as different dopamine-containing cells. As laid out in the preceding sections, there are several different cell sources that have demonstrated a capacity to survive and reverse motor alterations in animal models of PD. However, the clinical benefits of brain grafting in PD patients have not yielded the expected results. A look back in history indicates that some questions related to basic aspects of molecular and cellular physiology, as well as neuronal network function, remain unanswered.

One important issue is to identify the factors that determine whether a graft will induce motor recovery or not. Independently of the cell type used, the available evidence shows that, in animal models or human subjects, some graft recipients exhibit no recovery despite having equivalent levels of graft survival to the individuals that presented striking motor improvement. The reason for this variability is still unknown. Some results have shown that electrical activity of the grafted cells is a common feature of those animals with compensated behavior[88]. But how is this electrical activity or integration of the grafted cells achieved? The question remains unanswered. One possibility is that the host needs to have one or more individual-specific traits to provide a permissive microenvironment for the correct integration of the graft into the host tissue. These traits may involve molecular and cellular signaling pathways and communication between the endogenous and exogenous cells. What are these traits? Are there genetic or immunologic factors involved? Knowing the answer to these questions would allow clinicians to predict who can be a candidate for cell-replacement therapy, or even adjust the microenvironment of a host or the nature of the grafted cells to successfully treat all PD patients in an individualized manner. Current technology can be used for answering these questions. For example, current genome engineering technology such as CRISPR-Cas (see[95] for review) and TetR-, Cre- or Flp-mediated DNA recombination (see[96] for review) could allow us to delete, insert, reverse, silence or enhance the expression of different genes in order to elucidate the factors involved in the permissibility of the host. This technology would also contribute to understanding the mechanisms and molecules involved in the communication between the cells from the graft and those from the host. Additionally, regarding the influence of the microenvironment on the grafted cells, it has been shown that uncommitted ESC-derived cells grafted into different areas of the brain are capable of sensing the host site, and respond by modifying their survival and differentiation into a specific cell type[97].

Another important aspect is to understand the mechanisms related to the functionality of the graft. The unanswered questions in this regard are more related to systems-biology aspects concerning the consequences of the graft on the basal ganglia circuit. Further studies are necessary to determine the physiological consequences of grafting over the altered basal ganglia connections during natural behavior, as opposed to the use of pharmacological tools. By combining current approximations such as *in vivo* electrophysiological recordings or optogenetic activation and calcium imaging, it would be possible to determine whether grafts have differential effects on the activity of the direct and indirect pathways of the basal ganglia, and in general over the dynamics of the striatal microcircuit. These technologies have been used for the study of the normal function of the basal ganglia circuit and have also been applied to animal models of PD (*e.g.*, Ref[98-100]). Additionally, by coupling *in vivo* pharmacology experiments with optogenetics[101], we can understand more about the mechanisms underlying the functionality of the grafts in PD, as has been done recently[93].

Survival of grafted dopaminergic neurons remain as a limitation; only 1% to 20% of FVM-derived cells are able to survive in animal models of PD[102]. Different cellular stress responses occurring by the dissection of the cells and after the graft procedure are part responsible for the observed cell death[102]. The majority of the studies that follow graft survival and behavior in animal models focus on analyzing short and medium periods of time (*e.g.*, Ref[64,102]). However, despite the low survival of grafted cells, clinical trials have shown cases with significant motor improvements that last for varying time periods (*e.g.*, over some years to 20 years after grafting of human mesencephalic tissue[103]). Thus, as long as the underlying mechanisms related to the variability observed between subjects is comprehended, controlled and reduced, transplantation of dopaminergic-containing cells could be a potential treatment for motor symptoms in PD.

Finally, we have to remember that PD is a very complex disease that affects other systems in addition to the dopaminergic pathway[104]. Thus, the aim of cell replacement therapy in PD is merely symptomatic, and focused exclusively on the motor symptoms associated with the degeneration of the nigrostriatal pathway. An important concern related to the pathology *per se* is the fact that some PD-grafted patients have shown Lewy-body inclusions in the grafted cells[105]. Lewy-bodies are aggregates of normal, misfolded and truncated proteins and ubiquitin enzymes mainly composed of α-synuclein, and constitute the histological hallmark of PD (see[106] for review). This discovery is part of the evidence that supports the idea that PD spreads as a prion-like pathology (see[107] for review). Thus, it is probable that independently of the site of grafting, striatum or SNpc, the grafted cells will eventually develop the pathology. However, as Petit, Olsson and Brundin[108] have argued, the observation of Lewy-body inclusions does not necessarily invalidate the cell replacement therapy approach, based on the following arguments: Some patients have demonstrated motor improvements for up to 18 years; only a small proportion of grafted cells present Lewy-body inclusions; and finally we have to examine the cost-effectiveness relationship. Despite the logic of the arguments, on which we agree, we still have to remember that cell replacement therapy is not a cure for the disease, but rather a symptomatic relief. Thus, understanding the mechanisms related to the pathophysiology of PD is of fundamental importance if we wish to provide a more definitive strategy to face this disease (for review see[109]).

CONCLUSION

Important progress has been made since the first demonstration of a functional effect of dopaminergic-cell grafts in an animal model of PD. After the first decade of cell grafting in PD, it was clear that FVM-derived cells

were a better cell source for grafting in comparison to chromaffin cells derived from the AM. To date FVM-derived cells are considered as the most promising source for cell therapy in PD. After all these years of extensive efforts, it has been demonstrated that striatal FVM grafts survive, extend projections, release dopamine and more importantly, alleviate motor alterations in both animal models and in human subjects with Parkinson's disease. Cell integration is also important for achieving a positive functional outcome in other cell sources such as ESC-derived dopaminergic neurons. In addition, midbrain dopaminergic neuron grafts placed directly into the SNpc have also been shown to survive, to extend projections into the striatum, to increase striatal dopamine content, and to induce functional recovery. These observations are important and encouraging as they point to the possibility of reconstructing the nigrostriatal dopaminergic pathway.

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ORIGINAL ARTICLE

Basic Study

Copper as an alternative antimicrobial coating for implants - An *in vitro* study

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Abstract

AIM

To investigate osteoconductive and antimicrobial properties of a titanium-copper-nitride (TiCuN) film and an additional BONIT® coating on titanium substrates.

METHODS

For micro-structuring, the surface of titanium test samples was modified by titanium plasma spray (TPS). On the TPS-coated samples, the TiCuN layer was deposited by physical vapor deposition. The BONIT® layer was coated electrochemically. The concentration of copper ions released from TiCuN films was measured by atomic absorption spectrometry. MG-63 osteoblasts on TiCuN and BONIT® were analyzed for cell adhesion, viability and spreading. In parallel, *Staphylococcus epidermidis* (*S. epidermidis*) were cultivated on the samples and planktonic and biofilm-bound bacteria were quantified by

counting of the colony-forming units.

RESULTS

Field emission scanning electron microscopy (FESEM) revealed rough surfaces for TPS and TiCuN and a special crystalline surface structure on TiCuN + BONIT®. TiCuN released high amounts of copper quickly within 24 h. These release dynamics were accompanied by complete growth inhibition of bacteria and after 2 d, no planktonic or adherent *S. epidermidis* were found on these samples. On the other hand viability of MG-63 cells was impaired during direct cultivation on the samples within 24 h. However, high cell colonization could be found after a 24 h pre-incubation step in cell culture medium simulating the *in vivo* dynamics closer. On pre-incubated TiCuN, the osteoblasts span the ridges and demonstrate a flattened, well-spread phenotype. The additional BONIT®-coating reduced the copper release of the TiCuN layer significantly and showed a positive effect on the initial cell adhesion.

CONCLUSION

The TiCuN-coating inhibits the formation of bacterial biofilms on orthopedic implants by influencing the "race for the surface" to the advantage of osteoblasts.

Key words: Implant-coating; Antimicrobial effect; Titanium plasma spray; Titanium-copper-nitride; BONIT®; Osteoconductivity

Core tip: Implant-associated infection is the most feared complication after joint replacement. We investigated the osteoconductive and antimicrobial properties of a titanium-copper-nitride (TiCuN) film and an additional BONIT® coating on titanium. TiCuN released high amounts of copper quickly within 24 h and after 2 d, no planktonic or adherent Staphylococcus epidermidis were found on these samples. A high colonization by osteoblast-like MG-63 cells was found after pre-incubation in medium for 24 h. TiCuN inhibits the formation of bacterial bio-films on orthopedic implants by influencing the "race for the surface" to the advantage of osteoblasts.

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INTRODUCTION

Materials commonly used for permanent implants such as knee and hip prostheses are for the most part inert. However, researchers have recently taken up the challenge of designing biomaterials which have been physically and/or chemically modified to promote the regenerative processes of the affected tissues[1-3]. Increased surface area (roughness) on implants improves bone-to-implant contact after the implant placement and enhances functional activity of bone cells in contact with the biomaterial[4-7]. Titanium is one of the most common materials used for orthopedic implants[8,9] and surface modifications are created by sandblasting, plasma spraying or etching to accelerate osseointegration[10].

Despite aseptic operation conditions and perioperative antibiotic prophylaxis, implant-associated infections remain one of the most severe complications after joint replacement[11-14], occurring even more frequently after revision arthroplasty[15]. *Staphylococcus epidermidis* (*S. epidermidis*) and *Staphylococcus aureus* are the most frequently found microorganisms causing such implant-associated infections. The pathogenesis of infections associated with biomaterials is as follows: After an initial, reversible adhesion of the bacteria, a biofilm is formed[16-18] which enables the bacteria to avoid immune responses and circumvent antibiotics[19]. Antimicrobial agents do not succeed as well against biofilm bacteria as against planktonic bacteria[19]. In addition, infected medical devices continue to pose problems in orthopedic surgery, thus warranting further development of effective prevention and treatment strategies, including the use of thin coatings based on metal-ions[20]. There are several metal ions (Cu^{2+}, Ag^{+}, Zn^{2+}) which are known to have antibacterial properties and which could be deposited on the surface of implants[21,22]. Silver, for example, has been in use as an antibacterial coating for medical devices[23-26]. However, the lower toxicity and higher cytocompatibility of copper commends this metal ion for deposition on implant surfaces[22]. Furthermore, copper can be metabolized[27], whereas silver tends to resist metabolization, increasing body's silver serum level[28]. Although the general antimicrobial effects of copper have been recognized, to date researchers have little experience with the use of copper as an antimicrobial agent on medical implant surfaces[29-31]. This lack of data on the effects of copper prompted us to study its qualities as an antibacterial agent in this context. We studied the effects of the deposition of a copper-based inter-metallic thin film on titanium plasma spray optimized (TPS) titanium substrates. Our particular interest was in finding a deposited film which exhibits an antimicrobial effect while allowing for sufficient growth and vitality of osteoblasts on the surface. Taking these two factors into account, we investigated the properties and effects of titanium-copper-nitride (TiCuN) films deposited by physical vapor deposition (PVD). For this purpose we studied the chemical composition of the coating and the release of copper from it, investigating its antibacterial properties and the influence on cell growth, as well as determining the influence of an additional osteoconductive coating with a BONIT® layer.

MATERIALS AND METHODS

Preparation of coatings and test samples

Commercially pure titanium (grade 5, DOT, Rostock, Germany) of technical purity was used in the form of cylindrical plates of 11 mm in diameter and 2 mm thick. For micro-structuring, the surface of the test samples was modified by TPS. For the TPS coating, argon is ionized in a high temperature plasma flame in a vacuum chamber. The argon gas heats up and expands rapidly being expelled at high speed through an anode. Simultaneously titanium powder is inserted into the plasma flame and the molten titanium particles adhere to the substrate surface, cool rapidly and fuse to the implant surface. On the TPS-coated titanium test samples, a TiCuN layer with an average copper load of 1-3 μg/mm² was deposited by PVD (DOT). Copper and titanium were released from a target by electricity, ionized and deposited on the sample surface. The procedure developed a face-centered cubic network of titanium atoms with nitrogen ions inserted in the gaps. The TiCuN coating is very thin and only modifies the implant surface, leaving the mechanical properties of the implant unchanged[32-35]. The second coating on the TiCuN-layered samples was a BONIT® layer (DOT) applied using an electrochemical process. Samples were packed into sterilization foils (Direct, Konstanz, Germany), sealed, and gamma-sterilized with a minimum dose of 25 kGy of Co-60 radiation (BBF Sterilisationsservice, Kernen-Rommelshausen, Germany).

We refer to these different samples as follows: TPS: Commercially pure titanium modified by TPS; TiCuN: TPS + TiCuN; TiCuN + BONIT®: TPS + TiCuN + BONIT®.

Characterization of the coatings

Roughness of the sample surfaces was analyzed by a Hommel tester (Hommel Etanic T 8000, Jenoptik, Jena, Germany). Coating thickness and porosity was determined according to the Standard Test Method for Stereological Evaluation of Porous Coatings on Medical Implants ASTM F 1854. Adhesive strength of the coatings was determined according to DIN EN 582 with the universal tensile testing machine Shimadzu AG-50KNG (Shimadzu, Kyoto, Japan). To investigate the surfaces of the different materials, samples were gold sputtered by a coater (SCD 004, BAL-TEC, Balzers, Liechtenstein) and the surfaces were examined by field emission scanning electron microscopy (FESEM, SUPRA 25, Carl Zeiss, Oberkochen, Germany).

Copper release measurement

The concentration of copper released from the samples was measured by atomic absorption spectrometry (AAS) (ZEEnit 650, Analytik Jena AG, Jena, Germany) with electro-thermal atomization as described earlier[36]. Briefly, the substrates were stored in 1 mL Dulbecco's modified Eagle medium (DMEM, Invitrogen, Darmstadt, Germany) with 10% fetal calf serum (FCS, Superior, Biochrome, Berlin, Germany) and 1% gentamicin (Ratiopharm, Ulm, Germany) at 37 ℃ in a humidified atmosphere with 5% CO_2. The copper concentration of this DMEM solution was measured after 24 h and after incubation for another 24 h on three samples each per coating method. Nitric acid was used to stabilize copper ions released in the DMEM after storage. The supernatant was diluted to 1:100000 and a volume of 20 μL of the diluted solution was used for analysis. The intensity measured was compared with the standard reference intensity to obtain the number of copper atoms released from the sample (n = 3). Copper release from samples seeded with MG-63 osteoblasts (see paragraph cell culture) was determined in the supernatant accordingly.

Investigations of antibacterial effects

Estimation of the antibacterial potential against *S. epidermidis* on test samples was completed according to the protocols described earlier[37,38]. The biofilm-forming strain of *S. epidermidis* (ATCC 35984, American Type Culture Collection, Manassas, VA, United States) was routinely cultured on Columbia blood agar plates (Thermo Fisher Scientific, Waltham, MA, United States). Previous to the test, an overnight culture (37 ℃, microaerobic conditions) of *S. epidermidis* was prepared in a tryptic soya broth medium (Sigma-Aldrich, St. Louis, MO, United States). Afterwards, the overnight culture was centrifuged at 4000 rpm for 10 min at 4 ℃, after a washing step the bacteria pellet was diluted in 1 × PBS and adjusted to its strain-specific OD at 600 nm to obtain 1 × 10^8 CFU/mL in tryptic soya broth medium. For the experiments, bacteria were diluted in DMEM containing 10% FCS until 1 × 10^3 CFU/mL was achieved. After 2 d of incubation at 37 ℃, 5% CO_2, *S. epidermidis* within the biofilm on the test samples were detached by ultrasonic treatment with a sonication bath for 4 min at 35 kHz (BactoSonic, Bandelin Electronic, Berlin, Germany) and deposited into glass test tubes (Greiner Bio-One, Kremsmünster, Austria) with 1 mL of PBS. Subsequently, the solution was serially diluted in PBS and afterwards plated on TSB-agar with the help of a spiral plater (Eddy Jet 2, IUL, S.A., Barcelona, Spain). After 24 h of incubation at 37 ℃, 5% CO_2, colony-forming units were determined. To analyze the planktonic, unbound *S. epidermidis,* supernatants of the test-samples were shifted into 15 mL centrifuge tubes (Greiner Bio-One) with 1 mL of PBS after 2 d of incubation. Supernatants were centrifuged at 4000 rpm for 10 min at 4 ℃ and diluted consecutively in PBS. To determine the quantity of colony-forming units, dilutions were plated on TSB-agar plates as described above (n = 6).

Cell culture

Human MG-63 osteoblast-like cells (ATCC, No. CRL-1427™, LGC Promochem, Wesel, Germany) were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% FCS and 1% gentamicin at 37 ℃ in a humidified atmosphere containing 5% CO_2. At subconfluency, cells were detached with 0.05% trypsin/0.02% EDTA (PAA Laboratories, Cölbe, Germany) for 5 min at 37 ℃.

Table 1 Characterization of the coatings

Coating	TPS + TiCuN	TiCuN + BONIT®
Coating thickness (μm)	200-400	10-30
Roughness Ra (μm)	30-60	-
Porosity (%)	20-40	60
Adhesive strength (MPa)	74	15

TPS: Titanium plasma spray; TiCuN: Titanium-copper-nitride.

After stopping the trypsinization by the addition of complete cell culture medium, an aliquot of 100 μL was put into 10 mL of CASY® ton buffer solution (Roche Innovatis, Reutlingen, Germany) and the cell number was measured in the counter CASY® Model DT (Schärfe System, Reutlingen, Germany). An appropriate cell number was seeded onto the samples as described for the following applications. Two different experimental arrangements were used: (1) the MG-63 cells were directly cultivated on the samples; and (2) to simulate the *in vivo* dynamics closer, the samples were pre-incubated in cell culture medium DMEM with 10% FCS and 1% gentamicin at 37 ℃ in a humidified atmosphere with 5% CO_2 for 24 h, then the medium was changed and the cells were seeded onto the surfaces and cultivated for another 24 h.

Cell viability

To study the influence of TiCuN on cell metabolism and vitality the MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Mannheim, Germany) was performed. Forty thousand cells were seeded onto the samples in 24-well plates either directly or on pre-incubated samples at a volume of 1 mL. After 24 h, the cell culture medium was replaced by 800 μL of fresh medium and 200 μL of the MTS solution and incubated for 3 h at 37 ℃ in a 5% CO_2 atmosphere. The spectrophotometric absorption of 5 × 100 μL of the culture medium of 3 samples was analyzed on a 96-well plate by an ELISA reader (Anthos 2010, Anthos Labtec Instruments, Wals-Siezenheim, Austria) at 490 nm (n = 3). The extinction is proportional to the number and the metabolic activity of the cells.

Flow cytometric measurement of cell adhesion

The cell adhesion of MG-63 osteoblasts on the different material surfaces was determined as already described[39]. Briefly, suspended MG-63 cells in DMEM with 10% FCS (5 × 10^4 cells/0.3 mL) were seeded directly onto sample discs. To avoid the seeding of cells beside samples, discs were laterally fixed in adhesive tapes (Carl Roth, Karlsruhe, Germany). After 10 min to allow cell sedimentation and adhesion to the surface, the supernatant containing the non-adherent cells was then drawn up with a pipette, transferred into 12 mm × 75 mm test tubes (BD Biosciences, Heidelberg, Germany) and analyzed by flow cytometry (FACSCalibur™; BD Biosciences). Cell adhesion of 3 independent experiments was then calculated in percent (n = 3).

Cell morphology and spreading

Material samples were pre-incubated in DMEM with 10% FCS and 1% gentamicin at 37 ℃ in a humidified atmosphere with 5% CO_2. After 24 h the medium was changed and 4.0 × 10^4 MG-63 cells were seeded onto the samples. After cultivation for 24 h, cells were washed with PBS, fixed with 4% glutaraldehyde (1 h, Merck, Darmstadt, Germany), dehydrated through a graded series of ethanol (30% 5 min, 50% 5 min, 75% 10 min, 90% 10 min, and 100% 2 × 10 min) and dried in a critical point dryer (K850, EMITECH, Cambridge, United Kingdom). Gold sputtering was performed with the coater (SCD 004, BAL-TEC). The morphology of the cells on the substrate surfaces was investigated by scanning electron microscopy (SEM, DSM 960A, Carl Zeiss). Spreading of the cells was quantified by ImageJ (Rasband, W.S., ImageJ, United States National Institutes of Health, Bethesda, Maryland, United States, http://imagej.nih. gov/ij/, 1997-2016). The cell area of 30 cells in 2 independent experiments was analyzed (n = 60).

Statistical analysis

The statistical significance was calculated using SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, United States). Data are expressed as mean values ± standard deviation (SD) and analyzed using Mann-Whitney U test or the t-test. Values were compared to TPS at the same time point and differences for all experiments were considered statistically significant at $P < 0.05$ ($^aP < 0.05$, $^bP < 0.01$, $^dP < 0.001$).

RESULTS

Sample characteristics

We tested TPS-coated titanium samples equipped with both a TiCuN layer and a BONIT® layer in order to determine their suitability as bone implants encompassing antimicrobial and osteoconductive characteristics. Samples were purchased from DOT Coating (Rostock, Germany). The characteristics of the different coatings are shown in Table 1.

Figure 1 shows FESEM images of the surfaces of the different samples. The visibly rough surface of the samples is caused by the titanium plasma spray technique for TPS and TiCuN. A special crystalline surface structure is visible on TiCuN + BONIT®. BONIT® is an absorbable composite layer of two thin crystalline calcium phosphate phases with different solubility, the more soluble outer calcium phosphate phase (brushite) and the inner crystalline hydroxyapatite phase (≥ 70% brushite and ≤ 30% hydroxyapatite). BONIT® was shown to promote a fast on growth of bone cells and bone formation on implant materials in earlier studies[40-42]. Therefore, we used this coating additionally on the TiCuN films to study the antimicrobial as well as osteoconductive properties combined in one sample.

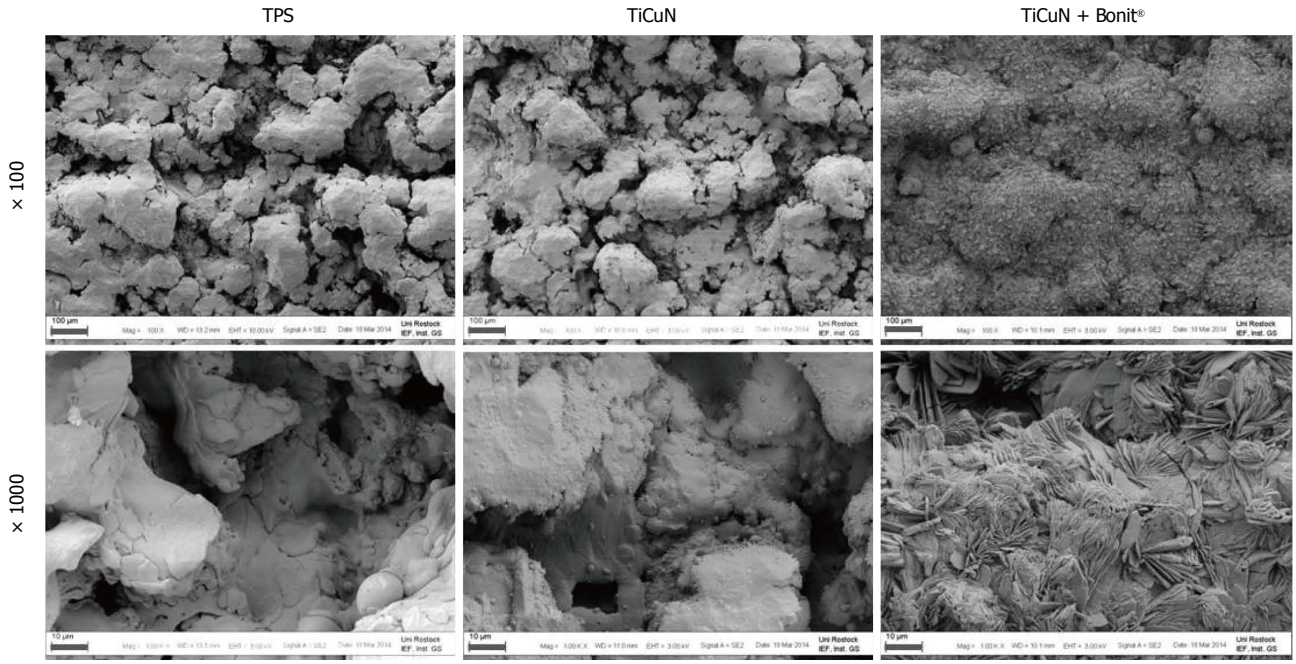

Figure 1 Surface topography of the coated materials *vs* titanium plasma spray control (field emission scanning electron microscopy, magnification × 100, × 1000, bars = 100 μm, 10 μm, respectively). TPS: Titanium plasma spray; TiCuN: Titanium-copper-nitride.

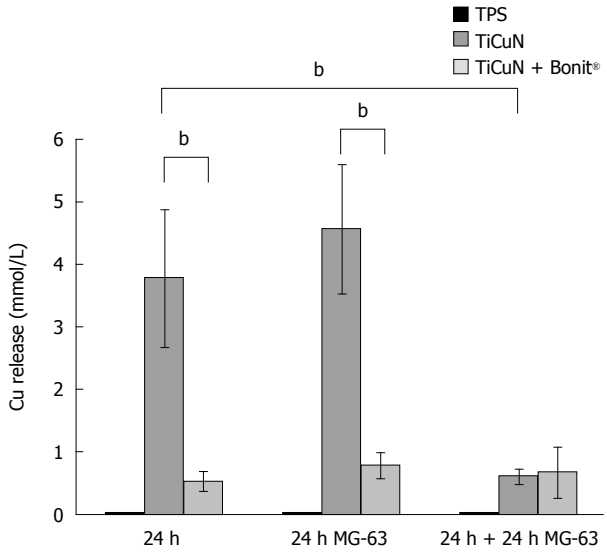

Figure 2 Copper release in Dulbecco's modified Eagle medium. A high amount of copper is released from the TiCuN layer after incubation in DMEM for 24 h. The copper release is reduced on TiCuN + BONIT® due to the BONIT® layer. A complete exchange of the medium and seeding with MG-63 cells for another 24 h reveals significantly reduced copper release from TiCuN. The amount is equalized to the level on TiCuN + BONIT® (n = 3, mean value ± SD, t-test, $^bP < 0.01$). TPS: Titanium plasma spray; TiCuN: Titanium-copper-nitride; DMEM: Dulbecco's modified Eagle medium.

Copper release

The results of copper release measurements from the samples in 1 mL of DMEM, indicated as mmol/L unit and dependent upon storage conditions, are shown in Figure 2. The highest copper release after 24 h was measured for TiCuN samples at about 3.8 mmol/L. Copper release was further elevated when samples were seeded with MG-63 osteoblastic cells and incubated for 24 h (around 4.6 mmol/L). For TiCuN samples which were pre-incubated in DMEM for 24 h and seeded with cells for another 24 h after exchanging the medium, copper release was significantly reduced to 0.6 mmol/L. TiCuN + BONIT® samples showed nearly constant low copper values between 0.5 and 0.8 mmol/L independently of the storage conditions. The BONIT® coating seems to slow down the release of copper from the TiCuN layer, resulting in a prolonged time of release.

Antibacterial effect

Heavy metal ions like copper ion can deactivate the central catabolic and biosynthetic pathways and become toxic[43]. We employed *S. epidermidis* strain RP 62A (ATCC35984) to study the influence of the TiCuN samples on the growth of bacteria. The antimicrobial effect of TiCuN films on *S. epidermidis* is presented in Figure 3. Only the TiCuN coating demonstrated growth inhibition; this indicates that the copper species was released into the medium at a high rate of diffusion. After 2 d, no planktonic or adherent *S. epidermidis* were found on the TiCuN samples. In contrast, the TPS discs proved to have 7.62×10^7 CFU/mL planktonic bacteria in the incubation fluids and 2.52×10^8 CFU/mL adherent bacteria in the rinsed fluids. The concentration of planktonic bacteria reached 1.08×10^8 CFU/mL in the incubation fluids from the TiCuN + BONIT® samples. An equal amount of biofilm-bound bacteria (1.33×10^8 CFU/mL) could be detected. Thus, no antibacterial potential was found after 24 h for TiCuN + BONIT®; it can be surmised that the low amount of copper released by this coating (between 0.5 and 0.8 mmol/L, see Figure 2) prevented any

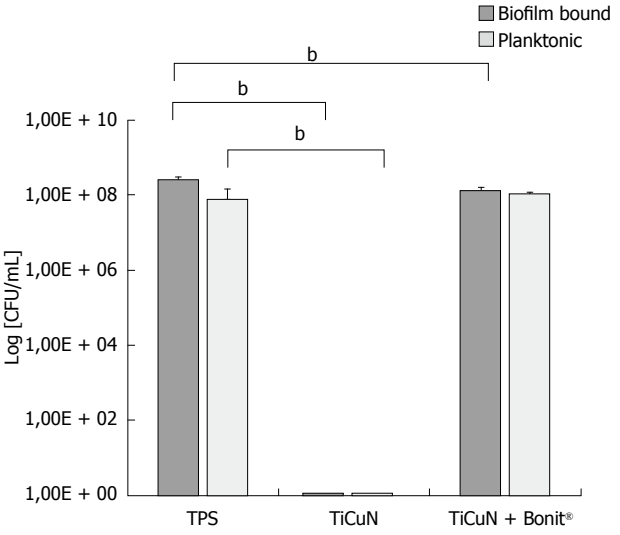

Figure 3 Antibacterial effect of the Titanium-copper-nitride coating on *Staphylococcus epidermidis* bacteria for planktonic and biofilm state after 2 d. On TiCuN, planktonic and biofilm bound bacteria were killed completely. On TiCuN + BONIT®, no antibacterial effect could be observed (n = 6, mean value ± SD, U-test, $^bP < 0.01$). TPS: Titanium plasma spray; TiCuN: Titanium-copper-nitride.

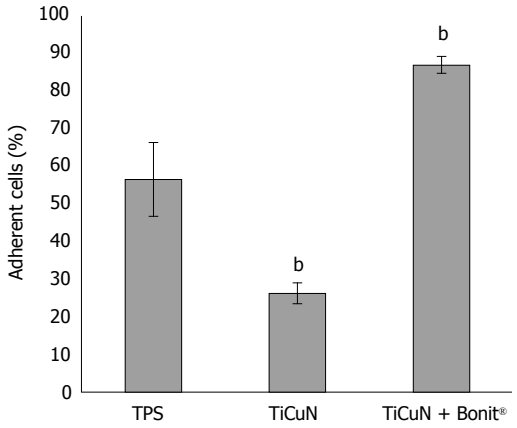

Figure 4 Initial cell adhesion of MG-63 osteoblasts on the titanium-copper-nitride. Surfaces compared to the titanium plasma spray control after 10 min. The MG-63 cells were directly seeded onto the samples and cultivated for 10 min. Cell adhesion was significantly reduced on TiCuN, but TiCuN + BONIT® enhanced cell adherence significantly (n = 3, mean value ± SD, t-test, $^bP < 0.01$). TPS: Titanium plasma spray; TiCuN: Titanium-copper-nitride.

significant antibacterial effect. The fast copper release from TiCuN samples can efficiently kill bacteria in the initial state of implantation and we assume that the risk of implant infection can thereby be significantly reduced.

Copper ions attack the bacteria at different sites[44-46]. They can interact with the outer membrane of bacteria and subsequently disintegrate the bacterial cell wall which is known as the bacteriolytic effect. If copper ions get into the bacteria, they can bind to the DNA and become involved in cross-linking within nucleic acid strands with the result that the bacteria cannot replicate. Furthermore copper ions generate reactive oxygen species and can cause lipid peroxidation and protein oxidation[47].

In addition, copper is an essential trace element present in many cell processes; a defect in the homeostasis of copper is a direct cause of certain human diseases[48]. Copper also plays a role in the control of cell proliferation[56]. Thus bioceramic scaffolds loaded with copper sulphate were shown to stimulate osteoblast activity and proliferation and the angiogenesis[49,50].

To determine the influence of the TiCuN and BONIT® coating on osteoblasts, we investigated the initial cell adhesion, the cell viability, the cell morphology and the cell spreading of MG-63 osteoblast-like cells after culturing on these surfaces.

Initial cell adhesion

Initial osteoblast cell adhesion was analyzed after 10 min of culturing (Figure 4). After direct seeding of MG-63 cells onto the samples, the non-adherent cells in the supernatant were measured by FACS. The adhesion of the cells was significantly reduced on TiCuN to about 26% compared to TPS where around 56% of the cells were adherent after 10 min. On the other hand, TiCuN + BONIT® enhanced initial cell adherence significantly (to about 87%).

Cell viability and spreading

The experiments to determine cell viability employed two different setups: (1) MG-63 cells were cultivated on the surfaces themselves; and (2) the samples underwent pre-incubation in cell culture medium DMEM for 24 h and cells were seeded onto the surfaces after a complete exchange of the medium. In this way the *in vivo* situation was simulated more closely, where dead cells and the persistent bacteria inside these cells are removed and new cells can adhere and proliferate on the surface. After incubation of the cells for 24 h, the cell viability was determined (Figure 5). Cultivation of the cells for 24 h directly on TiCuN reduced cell viability of the MG-63 cells to about 10% and on TiCuN + BONIT® for the same period to about 29% compared to TPS. Cells on TiCuN + BONIT® showed higher viability in comparison with TiCuN. This corresponds with the lower copper release values on these samples due to the BONIT® coating. Interestingly, the incubation of TiCuN samples for 24 h in DMEM prior to cultivating the cells led to an increase in cell viability by about 30%. During the pre-incubation period, a substantial amount of copper is released from the TiCuN film (Figure 2), after which the cells are able to grow onto the substrate surface. Although present, this effect is not as pronounced for TiCuN + BONIT®: Here, cell viability is increased by only 10%. So, on both samples cell viability reached around 40%. This corresponds to the low copper release measured on TiCuN and TiCuN + BONIT® after pre-incubation (between 0.6 and 0.7 mmol/L). The copper amounts released are slightly higher than the copper concentration limit identified for cell survival in earlier studies[51,57]. These studies showed that cell proliferation of hMSC is stimulated by copper concentrations below 0.3 mmol/L, whereas cell viability decreases significantly to around 30% at copper concentrations higher than

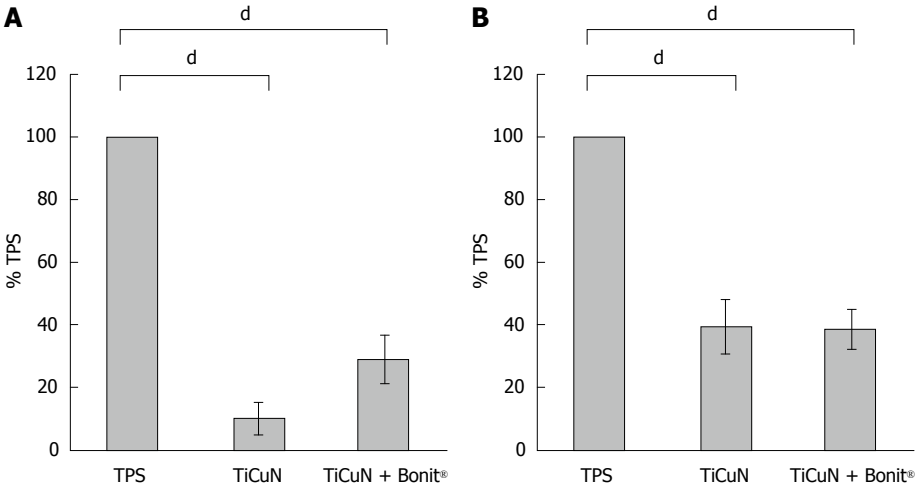

Figure 5 Viability of MG-63 osteoblasts on the titanium-copper-nitride surfaces. Two different experimental arrangements were used: (A) the MG-63 cells were directly cultivated on the TiCuN surfaces for 24 h and (B) the samples were pre-incubated in DMEM for 24 h and after this the cells were seeded onto the surfaces for another 24 h. Cell viability was significantly reduced after direct seeding on TiCuN. Cell viability was higher on TiCuN + BONIT® compared to TiCuN. Pre-incubation of the samples in DMEM for 24 h before seeding elevated cell viability on both samples (n = 3, mean value ± SD, t-test, $^{d}P < 0.001$). TPS: Titanium plasma spray; TiCuN: Titanium-copper-nitride; DMEM: Dulbecco's modified Eagle medium.

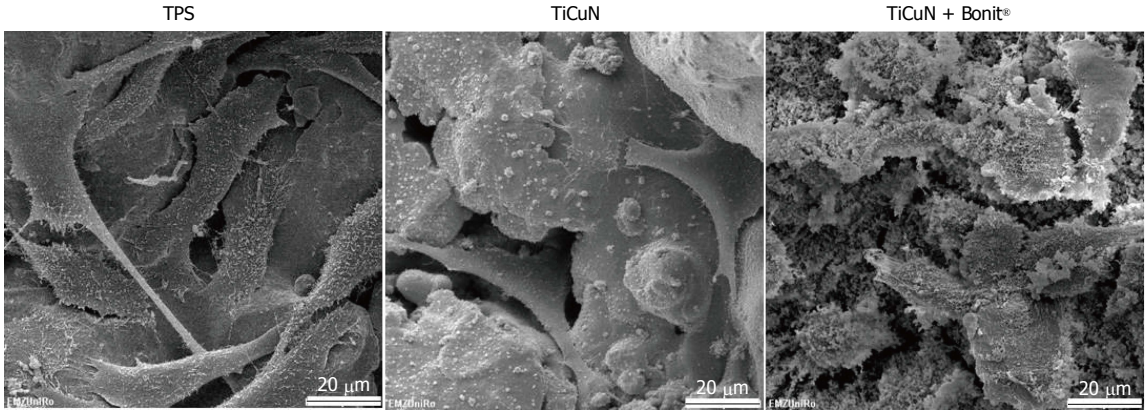

Figure 6 Scanning electron microscopy images of MG-63 osteoblasts on the pre-incubated surfaces. Samples were pre-incubated in DMEM for 24 h. After a complete exchange of medium, cells were seeded onto the surface and cultivated for another 24 h. Cells spread well on TPS and TiCuN surfaces but seem to be smaller on TiCuN + BONIT® (magnification × 1000). TPS: Titanium plasma spray; TiCuN: Titanium-copper-nitride; DMEM: Dulbecco's modified Eagle medium.

0.5 mmol/L.

Figure 6 shows SEM images of the osteoblasts growing on the sample surfaces**.** MG-63 osteoblasts were seeded onto the pre-incubated samples and cultivated for 24 h. It can be seen that the osteoblasts on the TPS reference and the TiCuN surfaces exhibit a flattened, well-spread phenotype and bridge the gaps between the ridges. The cells spread less readily on TiCuN + BONIT® and seem to be covered by small crystals evolved from the BONIT® layer. This is understandable, considering that BONIT® consists of a brushite and a hydroxyapatite phase. The more soluble brushite is metastable at a physiological pH and converts to a less soluble apatite phase[52,53]. During this phase transformation, loose crystal particles are released onto the settled cells and the surface cannot be considered solid. This explains the reduction in cell area on TiCuN + BONIT® compared to TiCuN and TPS, as revealed by the statistical analysis (Figure 7).

DISCUSSION

Our cell biological investigation revealed a cytotoxic effect on osteoblasts within 24 h by the TiCuN coating. On the other hand, the TiCuN surface showed a strong antibacterial influence on both planktonic and biofilm-bound *S. epidermidis*. The BONIT® coating reduced the copper release significantly within 24 h and as a consequence, no antibacterial effect could be demonstrated on TiCuN + BONIT® samples. The viability of osteoblasts on the TiCuN samples could be enhanced by a pre-incubation step. The copper-coated materials and controls were incubated in cell culture medium for 24 h and cell seeding was performed after a complete exchange of the medium. In this way the *in vivo* dynamics were simulated: Dead cells and the persistent bacteria inside these cells are removed and new cells can adhere and proliferate on the surface. Using this approach the osteoblasts were able to grow

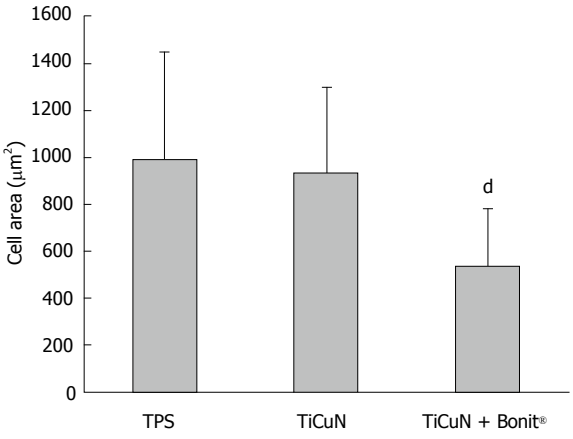

Figure 7 Spreading of MG-63 osteoblasts on pre-incubated samples after 24 h. Cell area is unchanged on TiCuN compared to TPS but significantly reduced on TiCuN + BONIT® due to the additional BONIT® layer (n = 60, mean value ± SD, t-test, $^dP < 0.001$). Titanium plasma spray; TiCuN: Titanium-copper-nitride.

properly. Stranak *et al*[36] found similar results for copper-doped titanium surfaces: Over a short period of time these released significant amounts of copper. Stranak *et al*[36] used dual high-power impulse magnetron sputtering which produced copper containing films on TiAlV alloys that released high amounts of copper (about 6 mmol/L) completely and quickly within 24 h. They were able to show an initial antibacterial effect within 24 h and high colonization by osteoblasts after replacement of the cell culture medium and cell seeding for another 24 h. A critical step in the development of implant-related infections is the surface adhesion of bacteria; this represents the first stage in the colonization process, the so-called "race for the surface" on the biomaterial[14,18]. Burghardt *et al*[57] demonstrated that complete killing of adherent bacteria within 24 h could be achieved by a final concentration of 1.75 mmol/L copper in the culture medium. The indicated bactericidal properties of copper can be used to hamper the settlement of an implant material by bacteria. It is, however, important to take into consideration the sensitivity to concentration displayed by copper's functional effects. It was found that copper acts as an antibacterial agent above concentrations of 0.5 mmol/L[51] and an osteoinductive one in the range of 0.05-0.3 mmol/L copper[57]. Therefore, it is suggested to use implants which initially introduce copper onto the surface at a high concentration to create an antibacterial effect in the vicinity of the implant. The stimulating effect on osteoblasts will prevail at a greater distance from the implant surface and later on. Some studies reported an additional advantage of depositing copper: It has lower toxicity and higher cytocompatibility compared to other antimicrobial metals. A relatively lower concentration of silver or zinc could have strong toxicity to the tissue cells; however, a relatively higher concentration of copper still had no toxic effect on the cells[22,27]. Further, copper represents an essential cofactor in collagen formation through its facilitation of the enzyme lysyl oxidase[54]. Recent studies which introduced copper combined with hyaluronan into elastin-vascular constructs were able to demonstrate increased synthesis of lysyl oxidase and collagen as well as stimulated elastin-crosslinking[55]. Various studies have shown the proliferation of human mesenchymal stem cells to be stimulated by copper ions; this makes the incorporation of copper into implant surfaces an interesting approach for tissue engineering in regenerative medicine[36,48,50,51,56,57]. In the study presented here we could show that TiCuN coating on TPS-optimized titanium combines a rough TPS surface with the antibacterial function of copper ions while maintaining the excellent properties required for good osteoblast cell growth. Our data were acquired by *in vitro* experiments, investigating processes within the first 48 h of material cell contact with osteoblast-like MG-63 cells. In future research, data will be verified by *in vitro* analyses after longer periods of time and with primary osteoblasts. In an animal study, we will examine the *in vivo* acceptance of the TiCuN and BONIT® coating on TPS-optimized titanium implants. Patients' first experiences provided in a clinical case report indicated that TiCuN-coated implants can be suitable as temporary spacers for two-stage septic joint revisions[31]. In conclusion, the TiCuN coating is indicated as a suitable method of reducing bacteria adhesion and promoting the growth of osteoblasts on implants. The additional BONIT® layer could be accomplished by another TiCuN coating or usage of an antibiotic to preserve the antibacterial effect and the osteoinductive influence.

In this study the antibacterial effect of TiCuN-coated, TPS-optimized titanium was examined. We showed that TiCuN has a strong ability to kill planktonic bacteria as well as bacteria adhering as a biofilm, and after pre-incubation we found low cytotoxicity. The antibacterial role should inhibit the formation of bacterial bio-films on orthopedic implants by influencing the "race for the surface" to the advantage of the osteoblasts.

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COMMENTS

Background

Titanium is one of the most common materials used for orthopedic implants. Increasing the roughness of the implant surface improves bone-to-implant contact after implant placement and enhances the functional activity of bone cells in contact with the biomaterial. Implant-associated infections remain one of the most severe complications after joint replacement. Bacteria interact with the surface of the material and after an initial reversible adhesion, a biofilm is formed. Such biofilms enable bacteria to evade antibiotics and immune responses.

Research frontiers

The problems associated with infected medical devices in orthopedic surgery necessitate further research and the development of alternative treatment and

prevention strategies, such as thin metal-ion based surfaces.

Innovations and breakthroughs

Some studies reported that copper represents a promising metal ion for deposition applications because of its lower toxicity and higher cytocompatibility compared to other antimicrobial metals. The authors investigated the properties and effects of titanium-copper-nitride (TiCuN) films deposited by physical vapor deposition. They studied the chemical composition and copper release with respect to antibacterial properties and cell growth and the influence of an additional osteoconductive coating with a BONIT® layer. The authors were able to show that a TiCuN coating on TPS-optimized titanium combines the rough TPS surface with the antibacterial function of copper ions, while maintaining the excellent properties required for good osteoblast cell growth.

Applications

In conclusion, the TiCuN coating is an interesting agent to inhibit the formation of bacterial bio-films on orthopedic implants by influencing the "race for the surface" to the advantage of the osteoblasts.

Peer-review

This is a very interesting topic and very well-presented scientific research. The study design is solid and meticulously and flawlessly conducted, the results of this study can be very important to professionals who perform these procedures.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Developing a donation after cardiac death risk index for adult and pediatric liver transplantation

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Author contributions: Khorsandi SE designed and wrote study; Giorgakis E performed the analysis and was involved in writing the manuscript; Vilca-Melendez H, O'Grady J, Heneghan M, Aluvihare V, Suddle A, Agarwal K, Menon K, Prachalias A, Srinivasan P, Rela M and Jassem W were all involved in the editing and review of the manuscript; Heaton N provided guidance in the manuscript composition and gave final institutional approval.

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Abstract

AIM

To identify objective predictive factors for donor after cardiac death (DCD) graft loss and using those factors, develop a donor recipient stratification risk predictive model that could be used to calculate a DCD risk index (DCD-RI) to help in prospective decision making on organ use.

METHODS

The model included objective data from a single institute DCD database (2005-2013, *n* = 261). Univariate survival analysis was followed by adjusted Cox-regressional hazard model. Covariates selected *via* univariate regression were added to the model *via* forward selection, significance level *P* = 0.3. The warm ischemic threshold was clinically set at 30 min. Points were given to each predictor in proportion to their hazard ratio. Using this model, the DCD-RI was calculated. The cohort was stratified to predict graft loss risk and respective graft survival calculated.

RESULTS

DCD graft survival predictors were primary indication for transplant (*P* = 0.066), retransplantation (*P* = 0.176), MELD > 25 (*P* = 0.05), cold ischemia > 10 h (*P* = 0.292) and donor hepatectomy time > 60 min (*P* = 0.028).

According to the calculated DCD-RI score three risk classes could be defined of low (DCD-RI < 1), standard (DCD-RI 2-4) and high risk (DCD-RI > 5) with a 5 years graft survival of 86%, 78% and 34%, respectively.

CONCLUSION

The DCD-RI score independently predicted graft loss (P < 0.001) and the DCD-RI class predicted graft survival (P < 0.001).

Key words: Liver transplant; Donor after cardiac death; Pediatric; Adult; Survival

Core tip: Calculating the donor after cardiac death (DCD) Risk Index score using objective variables from the donor (cold ischemic time, warm ischemic time, donor hepatectomy time) and from the selected recipient (primary indication for transplant, model for end-stage liver disease, retransplantation) can help rationalize the risk of using a DCD liver in a given recipient in order to produce good results.

Khorsandi SE, Giorgakis E, Vilca-Melendez H, O'Grady J, Heneghan M, Aluvihare V, Suddle A, Agarwal K, Menon K, Prachalias A, Srinivasan P, Rela M, Jassem W, Heaton N. Developing a donation after cardiac death risk index for adult and pediatric liver transplantation. *World J Transplant* 2017; 7(3): 203-212 Available from: URL: http://www.wjgnet.com/2220-3230/full/v7/i3/203.htm DOI: http://dx.doi.org/10.5500/wjt.v7.i3.203

INTRODUCTION

The continuing organ shortage combined with an expanding transplant waiting list is the main determinant of death on the waiting list. From UNOS and Eurotransplant data, death on the waiting list stands at 12% and 27% respectively[1,2]. This has driven the need and usage of the marginal liver or extended criteria organ, a term that encompasses the donor after cardiac death (DCD) liver. DCD liver transplantation has grown exponentially in countries that have utilized this form of donation[3]. Institutionally, DCD donation accounts for over 20% of transplants performed[4]. However, reports of poor patient and graft survival highlight that this is an organ with risks. A number of different factors have been identified as contributing to good outcome after DCD liver transplantation including donor factors of age, cold ischemic time (CIT), warm ischemic time (WIT), donor weight > 100 kg[5-8] and recipient factors of retransplantation (reTPL), on Intensive Therapy Unit (ITU) at time of transplant or renal dysfunction. Additionally, the use of a low risk DCD into a low risk recipient, can produce a good outcome that is equivalent to donor after brainstem death (DBD) liver transplantation[5].

Balancing the risk in DCD liver transplantation to achieve good results is still poorly understood and is both subjectively and experience driven. The aim of this study was to identify objective predictive factors for DCD graft loss and to use these factors to develop a donor recipient stratification risk predictive model that could be used to calculate a DCD risk index (DCD-RI) score to help in prospective decision making on DCD use.

MATERIALS AND METHODS

DCD practice and definitions

Institutionally the DCD programme started in 2001 and practice has been relatively consistent. In brief, recipient eligibility for DCD liver transplantation is decided at the liver transplant listing multidisciplinary meeting. Recipients offered a DCD liver are typically primary transplants for chronic liver disease (CLD) +/- hepatocellular cancer (HCC). The DCD liver was normally avoided in acute liver failure (ALF) or where a prolonged/difficult recipient hepatectomy was anticipated such as redo transplantation, young adult extrahepatic biliary atresia or the presence of an extensive portomesenteric venous thrombosis, as it would be anticipated to add to the CIT. Consent specifically for DCD transplantation would be obtained from the recipient.

For procurement a modified super rapid Casavilla technique was used[9]. Withdrawal of the DCD donor would either occur in the anaesthetic room or ITU depending on donor hospital preference. After declaration of cardiac death, there would be a 5 min stand off before the donor was brought into the operating room, where the donor team would be scrubbed and ready. After making a thoraco-abdominal incision, venting of blood would be in the chest, followed in sequence, by aortic cannulation, cross clamp in the chest and then portal/superior mesenteric vein cannulation. Adherence to WIT limits was consistent and a DCD liver would be discarded if the WIT exceeded 30 min[10]. The WIT was defined as the time from systolic of 50mmHg or oxygen saturations of 70%, depending on which agonal donor observation occurred first, to time of aortic cannulation.

Once perfusion had started, the gall bladder would be flushed until clear of bile followed by copious *in situ* flushing of the bile duct with chilled (4 ℃) normal saline. Topically, sterile crushed ice would then be placed around the organs to be retrieved. For perfusion *in situ* 4 L (aortic) and 2 L (portal) University of Wisconsin (UW) with 20000 IU heparin/L would be used. Pressure bags would only be used if flow by gravity was not sufficient. Attention to rapid donor hepatectomy was encouraged. On the back bench the portal vein (500 mL), hepatic artery (250 mL) and bile duct (250 mL) would be flushed further with chilled UW. Finally, the organ would be bagged for cold static storage.

Before proceeding with transplant the liver would be assessed on the backbench by the implanting surgeon. A severely steatotic liver on visual inspection would be discarded. If need be, a fresh frozen trucut liver biopsy

would be taken to assess degree of steatosis or to exclude donor pathology. DCD liver steatosis > 30% led to non-usage. Implantation technique was typically piggyback with a temporary portocaval shunt. The majority of livers were re-perfused *via* the portal vein. Standard immunosuppression was calcineurin inhibitor (tacrolimus) and steroid based. The cold ischemic time was the time from aortic cannulation in the donor to reperfusion in the recipient. Donor hepatectomy time (dHepT) was from the start of donor aortic perfusion to completion of hepatectomy. Model for end-stage liver disease (MELD) was defined as laboratory MELD and exception points have not been applied. The diagnosis of primary ischemic cholangiopathy (PIC) was based on review of biliary imaging by two consultant radiologists that demonstrated diffuse intrahepatic stricturing with no associated hepatic artery thrombosis (HAT).

Patient population and statistical analysis

The data analysed was extracted from a prospectively populated DCD database of a single institute, with a minimum follow up of 2 years (January 2005 - January 2013, n = 261). The pediatric age group was ⩽ 16 years. The study had full ethical approval in accordance with the declaration of Helsinki. Descriptive statistics were calculated for objective variables of the donor and the recipient, and for the calculated DCD-RI score. The developed DCD-RI model only included objective donor and recipient data. So subjectively assessed factors, such as liver steatosis were excluded from the analysis and the model. The primary end point was DCD graft loss. Survival analysis was performed using a Cox proportional hazard model and Kaplan-Meier estimator. Donor and recipient variables were tested independently to assess their uncontrolled effect on DCD graft survival. Significant predictive factors were then further analyzed separately with Kaplan-Meier and their respective ranges adjusted according to their level of significance. Similarly, primary indication for liver transplant was divided into 3 groups of high, standard and low risk according to their representation on the Kaplan-Meier survival curves.

For the development of the prediction model, etiology of liver disease was used as first indicator, which was then controlled for selected variables. Variables were added to the Cox regression model using forward selection with a significance level entry set at P = 0.3. Points were given to each variable in proportion to their calculated hazard ratio. WIT threshold was clinically set at 30 min and retained in the model. Using this model, the DCD-RI score was calculated for the study DCD cohort (n = 261). The DCD cohort was then stratified according to predicted graft loss risk as defined by the calculated DCD-RI score into three risk classes of low, standard and high. Respective predicted graft survivals were then calculated using Kaplan-Meier. Internal validation of the developed DCD-RI score was undertaken by performing a retrospective analysis on an earlier DCD cohort n = 37 (04/2001-12/2004), the experience of which has been previously published[11]. The receiver operator curve (ROC) and the area under the curve (AUROC) or c-statistic were then calculated to assess the performance of the DCD-RI score. The DCD-RI ROC curve was also compared to other scoring systems that have been used to predict graft survival after transplant. Statistical analysis was performed using SPSS® IBM® Statistics V22.0.

Table 1 Summary of the descriptive statistics for donor (d) and the recipient (r) that form the study donor after cardiac death cohort from which the donor after cardiac death risk index score was developed

DCD donor and recipient variables			All (n = 261)
Donor	dAge (yr)		46.1 ± 17.9
	dBMI		26 ± 4.9
	ITU Stay (d)		3.9 ± 5.8
	COD (CVA: Other: HBI: Trauma)		52.5:13.8:16.9:16.9
	dSodium (mmol/L)		144.51 ± 11.8
	dBilirubin (μmol/L)		9.81 ± 6.88
	Split/reduced (%)		2.30%
	WIT (min)		16.7 ± 9.8
	dHepT (min)		24.3 ± 10.6
	Liver Weight (g)		1518.28 ± 397.507
	CIT (min)		431 ± 118
Recipient	rAge (yr)		49.45 ± 15.36
	rGender		70.1%M/39.9%F
	rBMI		25.9 ± 4.7
	ALF (%)		1.50%
	rBilirubin (mmol/L)		89.36 ± 116.38
	rINR		1.89 ± 1.88
	MELD		14.8 ± 6.4
	Location (inpatient/home)		20.3%/79.6%
	Prior abdominal surgery (yr)		13.40%
	reTPL (yr)		5.70%
	Indication for TPL	Low	68 (26%)
		Standard	176 (67.5%)
		High	17 (6.5%)

Data presented as mean ± SD or % where appropriate. Primary indication for transplant has been divided into three risk groups of low, standard and high risk, as defined by their survival curves. BMI: Body mass index; COD: Cause of death; CVA: Cerebrovascular accident; HBI: Hypoxic brain injury; WIT: Warm ischemic time; CIT: Cold ischemic time; dHepT: Donor hepatectomy time; ALF: Acute liver failure; MELD: Model for end stage liver disease; reTPL: Retransplantation; TPL: Transplant.

RESULTS

Descriptive statistics for studied DCD transplant population

Table 1 summarizes the objective donor and recipient variables for the DCD study cohort (n = 261). The mean DCD recipient age was 49.45 ± 15.36 years, of which 15 (5.7%) were ⩽ 16 years. The mean DCD donor age was 46.1 ± 17.9 years, of which 18 (6.9%) were in the pediatric age group. Redo liver transplantation (reTPL) was a small component of the DCD programme accounting for 3.4% of activity in the period of study. The DCD liver was only used in a few cases of ALF (1.5%) and split/reduction (2.3%) was uncommon (Table 1). In the DCD study cohort the incidence of primary non

DCD risk group	Liver disease indication for transplant	Graft survival (%)					*P* value
		3 mo	6 mo	1 yr	3 yr	5 yr	
Low (*n* = 68)	AIH, PSC, NASH, HBV, cholestatic	96	94.5	91	89	86	0.066
Standard (*n* = 176)	Metabolic	91	89	85	79	76	
High (*n* = 17)	ALD, HCC, HCV, cryptogenic, other	87	84.5	76	69.5	64.5	

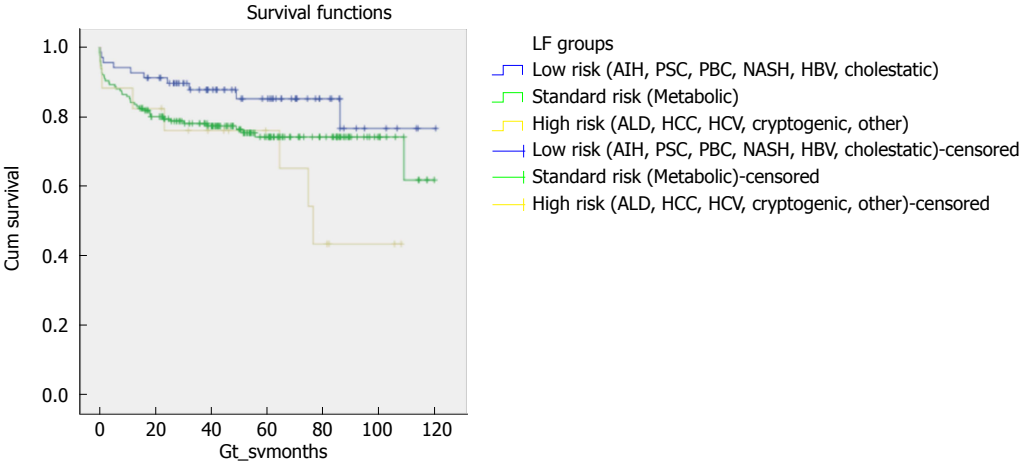

Figure 1 Stratified Kaplan-Meier curves for the cumulative donor after cardiac death graft survival in relation to primary indication for transplant and respective 3 mo, 6 mo, 1 year, 3 years and 5 years survival (χ^2 5.1 log-rank, *P* = 0.066). This stratification of indication for transplant defining the three risk groups of low, standard and high. Low DCD risk indications for transplant included autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), non-alcoholic steatohepatitis (NASH), hepatitis B virus (HBV) and cholestatic liver disease (primary familial intrahepatic cholestasis, extrahepatic biliary atresia and Crigler Najjar). Standard risk indications were metabolic diseases that included Wilson's, Hemochromatosis and Familial Amyloid Polyneuropathy. High risk indications for DCD transplant were alcohol related liver disease (ALD), hepatocellular carcinoma (HCC), hepatitis C virus (HCV), cryptogenic and Budd Chiari.

function (PNF) was 3.4% (*n* = 9), HAT 5% (*n* = 13), anastomotic biliary stricture 11.1% (*n* = 29) and PIC 3.5% (*n* = 9). Overall, there were 15% (*n* = 39) deaths and 3.5% (*n* = 9) retransplants.

Univariate analysis of donor and recipient risk factors for DCD graft loss

Univariate analysis of independent donor and recipient variables was initially performed to determine which variables were associated with DCD graft loss. Recipient variables analyzed were age (rAge), gender (rGender), weight (rWeight), BMI (rBMI), MELD, primary indication for transplant, patient location (home/hospital), reTPL, prior abdominal surgery and ALF/CLD. The donor variables analyzed were age (dAge), weight (dWeight), BMI (dBMI), cause of death (COD), sodium, CIT, WIT, liver weight, hepatectomy time (dHepT) and length of ITU stay (see Table 1).

On univariate analysis, the donor and recipient variables that were found to have a significant effect on DCD graft survival were MELD > 25 (χ^2 3.8 log-rank *P* = 0.05) and dHepT > 60 min (χ^2 4.8 log-rank *P* = 0.028). The variables that reached the significance level of entry into the forward selection regression model (*P* = 0.3) were primary indication for liver transplant (χ^2 5.1 log-rank *P* = 0.066), reTPL (χ^2 1.8 log-rank *P* = 0.176) and CIT > 10 h (χ^2 1.1 log-rank *P* = 0.292).

On grouping of survival curves based on primary liver disease indication for transplant, three DCD risk groups were defined of low, standard and high. Better survival was demonstrated when a DCD liver was used in a low risk indication for transplant (86% graft survival at 5 years) and poorer survival was found when the DCD liver was used in a high risk indication for transplant (64.5% graft survival at 5 years) (Figure 1). Low DCD risk indications for transplant included autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC), non-alcoholic steatohepatitis (NASH), hepatitis B virus (HBV) and cholestatic liver disease. The cholestatic low risk indications for transplant encompassed primary familial intrahepatic cholestasis (PFIC), extrahepatic biliary atresia (EHBA) and Crigler Najjar. The standard risk indications for transplant were metabolic diseases that included Wilson's, Hemochromatosis and Familial Amyloid Polyneuropathy. The high risk indications for DCD transplant were alcohol related liver disease (ALD), HCC, hepatitis C virus (HCV), cryptogenic and Budd Chiari. Survival analysis for these three DCD risk groups as defined by primary indication for transplant is illustrated in Figure 1 (χ^2 5.1 log-rank *P* = 0.066).

In the period of study, the use of DCD for reTPL was rare, 5.7% (*n* = 9). In the cases that DCD was used for reTPL, there was significantly worse DCD graft survival. At 5 years DCD graft survival in reTPL was 65% compared to 78% when used in primary liver transplant (see Figure 2 for survival curves, χ^2 1.8 log-rank *P* = 0.176). Use of DCD in recipients with higher MELDs ⩾ 26 (*n* = 11) was also found to be associated with worse DCD graft survival (χ^2 3.8 log-rank *P* = 0.05), with a 5-year survival of 56% compared to 78%, when used in recipients with a MELD < 25 (Figure 3 for DCD survival curves according to

DCD-RI independent variable	Graft survival (%)					*P* value
	3 mo	6 mo	1 yr	3 yr	5 yr	
reTPL						
Yes (*n* = 9)	91	78	78	65	65	0.176
No (*n* = 252)	91	90	87	82	78	

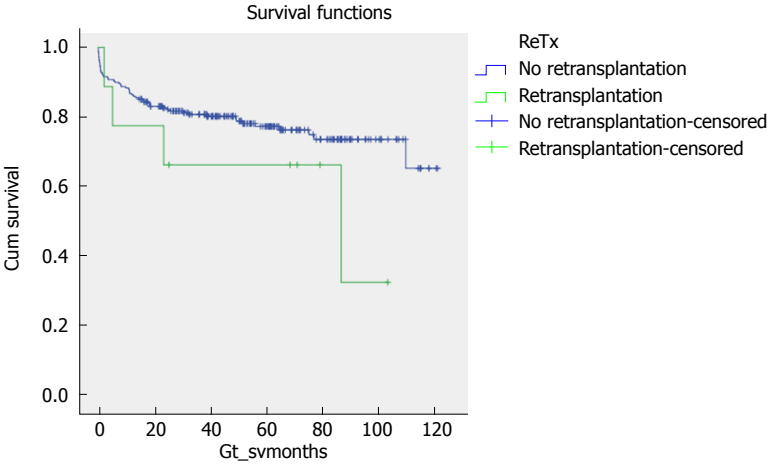

Figure 2 Stratified Kaplan-Meier curves for the cumulative DCD graft survival in relation to use in retransplantation or not and respective 3 mo, 6 mo, 1 year, 3 years and 5 years survival rates (χ^2 1.8 log-rank, *P* = 0.176).

MELD). Additionally, the donor hepatectomy time (dHepT) was found to be a determinant of DCD graft survival (χ^2 4.8 log-rank *P* = 0.028), with a dHepT ⩾ 60 min associated with early graft loss and a poor 5 year graft survival of 32% (Figure 4 for survival curves according to the dHepT groups).

Clinically, the warm ischemic threshold was set at 30 min and the WIT was retained in the DCD-RI model, despite not being found significant on univariate analysis, as it is institutionally regarded as a constant variable in determining outcome in DCD transplantation. After serial Kaplan-Meier analysis the CIT threshold was statistically set at ⩾ 10 h (*n* = 13) and < 10 h (*n* = 248) (χ^2 1.1 log-rank *P* = 0.292). However, many programmes are more stringent aiming for shorter CIT < 8 h (17). For the WIT, Kaplan-Meier analysis produced a cut off value of 25 min (*n* = 240) that had the lowest *P* value (χ^2 0.589 log-rank *P* = 0.443) and was the value incorporated into the developed DCD-RI model.

There was no difference in DCD graft survival between adult (*n* = 243) and pediatric (*n* = 18) donors (HR = 0.819, CI: 0.343-1.958, *P* = 0.653). Similarly, there was no difference in DCD graft survival between adult (*n* = 246) and pediatric (*n* = 15) recipients (HR = 1.268, CI: 0.389-4.132, *P* = 0.699). Therefore for the developed DCD-RI model adult and pediatric age groups have been combined.

Multivariate analysis and defining the DCD-RI score

Using primary indication for liver transplant as the primary indicator adjusted for the identified donor (WIT, CIT, dHepT) and recipient variables (MELD, reTPL) multivariate Cox regression analysis was undertaken. For the DCD-RI model points were given to each variable in proportion to the calculated hazard ratio (Table 2). According to the DCD-RI score three DCD-RI risk classes were defined, low risk (DCD-RI < 1), standard risk (DCD-RI 2-4) and high risk (DCD-RI > 5). Transplantation with a high risk DCD-RI score > 5 produced a 1 year graft survival of 40% and at 5 years 34% (Figure 5 for the survival curves according to DCD-RI score, Log-Rank and

Table 2 Point allocation system for the donor after cardiac death risk index score

Donor/recipient predictor variables	HR (CI)	Points
Primary indication for transplant		
Low (*P* = 0.07)		
Standard (*P* = 0.05)	2 (1-4.04)	2
High (*P* = 0.04)	2.83 (1.04-7.24)	3
reTPL (*P* = 0.26)	1.87 (0.63-5.58)	2
MELD > 25 (*P* = 0.04)	2.75 (1.04-7.24)	3
CIT > 10 h (*P* = 0.6)	1.37 (0.4-4.04)	1
WIT > 25 min (*P* = 0.4)	1.48 (0.6-3.63)	1
dHepT		
40-60 min (*P* = 0.5)	1.36 (0.53-3.53)	1
> 60 min (P = 0.05)	4.4 (1.02-19.04)	4

Points were given to each variable in proportion to their calculated hazard ratio (HR). Primary indication for liver transplant has been divided into three risk groups of low, standard and high, as defined by their survival curves. Low DCD risk indications for transplant include autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis, non-alcoholic steatohepatitis, Hepatitis B virus and cholestatic liver disease (primary familial intrahepatic cholestasis, extrahepatic biliary atresia and Crigler Najjar). Standard risk indications were metabolic diseases that included Wilson's, Hemochromatosis and Familial Amyloid Polyneuropathy. High risk indications for DCD transplant were alcohol related liver disease; HCV: Hepatitis C virus, cryptogenic and Budd Chiari. reTPL: Retransplantation; MELD: Model for end stage liver disease; CIT: Cold ischemic time; WIT: Warm ischemic time; dHepT: Donor hepatectomy time; HCC: Hepatocellular carcinoma.

DCD-RI independent variable	Graft survival (%)					*P* value
	3 mo	6 mo	1 yr	3 yr	5 yr	
MELD ⩾ 26 Yes (*n* = 11)	82	82	72	56	56	0.05
MELD ⩽ 25 No (*n* = 11)	92	90	83	81	78	

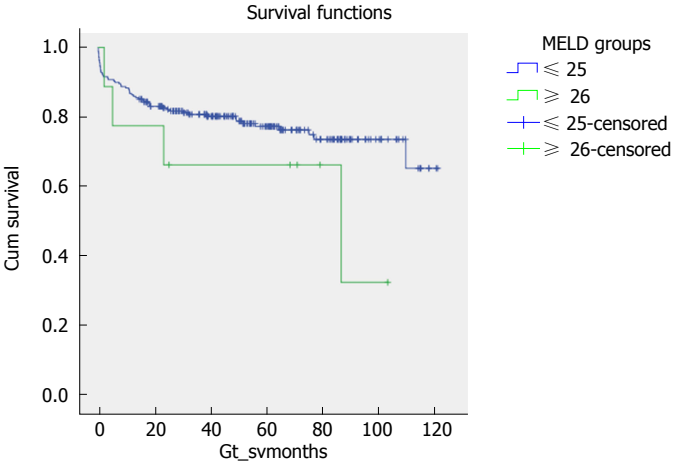

Figure 3 Stratified Kaplan-Meier curves for the cumulative DCD graft survival in relation to MELD and respective 3 mo, 6 mo, 1 year, 3 year and 5 year survival rates (χ^2 3.8 Log-Rank, *P* = 0.05).

DCD-RI independent variable	Graft survival (%)					*P* value
	3 mo	6 mo	1 yr	3 yr	5 yr	
dHepT						
⩽ 39 min (*n* = 237)	93	91	88	81	78	0.028
40-59 min (*n* = 21)	81	81	81	77	77	
⩾ 60 min (*n* = 3)	99	32	32	32	32	

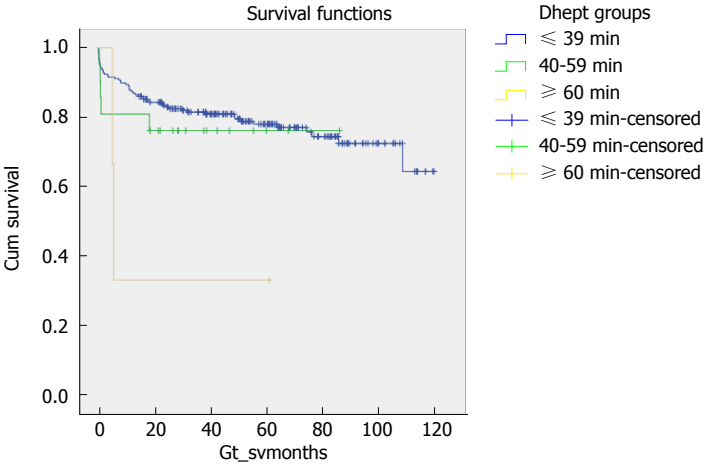

Figure 4 Stratified Kaplan-Meier curves for the cumulative DCD graft survival in relation to DCD donor hepatectomy time (dHepT) and respective 3 mo, 6 mo, 1 year, 3 years and 5 years survival rates (χ^2 4.8 Log-Rank, *P* = 0.028).

Breslow pooled over strata *P* < 0.001).

DCD-RI score internal validation

For internal validation of the developed DCD-RI score a retrospective analysis of an earlier DCD cohort 04/2001-12/2004 *n* = 37 were undertaken. Table 3 for summary of actual and predicted survival using the DCD-RI class subdivision. There was good concordance between actual graft survival and predicted DCD-RI survival, with actual graft survival falling within the confidence interval of the DCD-RI risk class predicted survival.

DCD-RI ROC and comparison to other predictive models

Based on the DCD-RI ROC, a DCD-RI score 1.5 cut off had a good positive predictive value (PPV = 0.993). A low risk DCD-RI score ⩽ 1.5, graft survival was predicted with 99.3% sensitivity and 98.3% specificity. Whereas, with a high risk DCD-RI score > 5, specificity was better than sensitivity (Figure 6 for DCD-RI ROC

DCD-RI risk class	DCD-RI score	Graft survival (%)					*P* value
		3 mo	6 mo	1 yr	3 yr	5 yr	
Low (*n* = 54, 20.6%)	0-1	96	95	93	89	86	0.000
Standard (*n* = 193, 74%)	2-4	93	90	87	82	78	
High (*n* = 14, 5.3%)	⩾ 5	80	75	40	34	34	

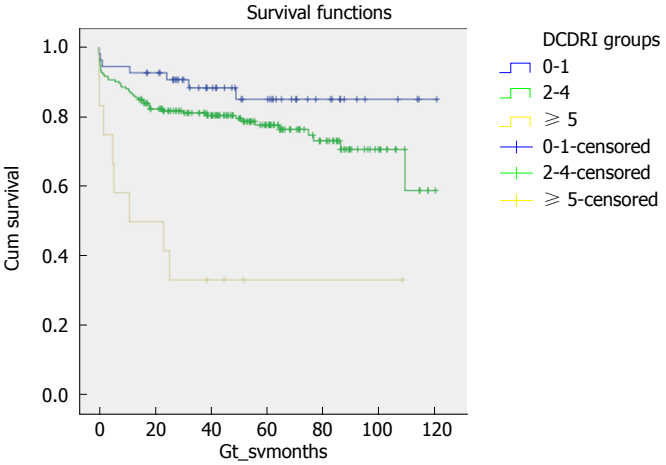

Figure 5 Stratified Kaplan-Meier curves for the cumulative DCD graft survival in relation to their DCD-RI score and respective 3 mo, 6 mo, 1 year, 3 years and 5 years survival (log-rank *P* = 0.000). The DCD-RI score divides the study cohort into three DCD-RI risk classes of Low (DCD-RI = 0-1), Standard (DCD-RI = 2-4) and High (DCD-RI ⩾ 5).

Table 3 Internal validation of the donor after cardiac death risk index in predicting donor after cardiac death graft survival

DCD Graft Survival (mo)	DCD-RI class		
	DCD-RI ⩽ 1, low (*n* = 10/27%)	DCD-RI 2-4, standard (*n* = 8/21.6%)	DCD-RI ⩾ 5, high (*n* = 19/51.4%)
3			
Actual	100	92.6	75
Predicted	96 (100-83.8)	90 (100-76.8)	80 (96.2-63.8)
6			
Actual	100	85.2	75
Predicted	95 (100-83.7)	90 (100-83.8)	75 (91.2-58.8)
12			
Actual	100	77.8	75
Predicted	93 (100-76.8)	87 (100-70.8)	40 (56.2-23.8)
60			
Actual	100	63	50
Predicted	86 (100-83.8)	78 (94.2-61.8)	34 (50.2-17.8)

The DCD-RI was calculated for an earlier DCD transplant cohort (2001 - 2004). The table summarizes actual and predicted graft survival as calculated with the DCD-RI. The DCD-RI predicted survival showed good correlation with actual graft survival, as actual graft survival fell within the confidence interval of DCD-RI predicted graft survival. DCD: Donor after cardiac death; DCD-RI: Donor after cardiac death risk index.

curve). To determine how the DCD-RI score compared to other transplant predictive scoring systems for graft outcome, the DCD-RI ROC curve was compared to other systems (see Figure 6). Based on the c-statistic (or AUROC) the DCD-RI (c-statistic = 0.657) was found to be better than MELD (c-statistic = 0.514) and better than the donor risk index (DRI) (c-statistic = 0.53) in predicting DCD graft loss when applied to the validation cohort.

DISCUSSION

The DCD liver is regarded as an extended criteria donor graft, in terms of the poorer outcomes that have been reported in the literature. Particular, concerns with this organ are the increased occurrence of PNF (0%-12%), early graft dysfunction (20%-30%), and PIC (15%) that result in the higher rates of graft loss and recipient death[5,6,12-18]. A large component in determining good results in DCD liver transplantation is the ability to balance risk through judicious matching of the donor and recipient. The aim of this work was to develop a formula, the DCD-RI that is valid and easy to apply with readily available objective variables relating to the donor and the recipient to help rationalize this risk balance.

The objective recipient variables that were found to have a significant effect on DCD graft survival were the primary indication for transplant, MELD and reTPL. While from the donor, CIT and hepatectomy time (dHepT) were important. These five variables combined with

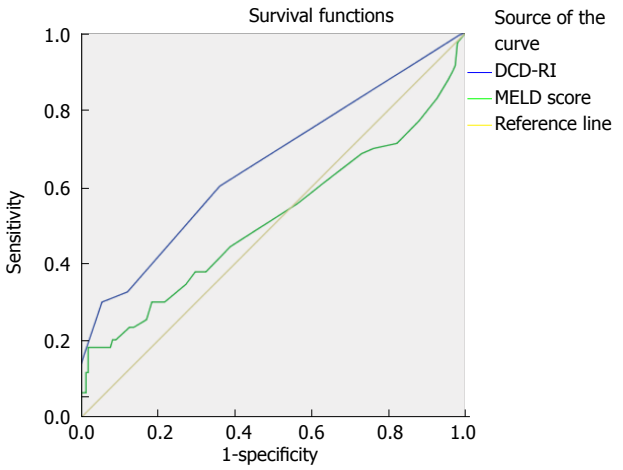

Figure 6 DCD-RI receiver operator curve and comparison to other predictive models illustrating that the DCD-RI performed better than model for end-stage liver disease in predicting graft survival. DCD-RI c-statistic = 0.657 and MELD c-statistic = 0.514). MELD: Model for end-stage liver disease; DCD-RI: Donor after cardiac death risk index.

the fundamental determinant of DCD graft outcome of WIT[7] formed the basis of the developed DCD-RI score. According to primary indication for transplant, three DCD risk groups of low, standard and high were defined (Figure 1). By applying this stratification, good graft survival of over 86% at 5 years was found when the DCD liver was used in recipients with low risk indications for transplant of AIH, PSC, PBC, NASH, HBV, and cholestatic diseases that included PFIC, EHBA and Crigler Najjar. While in the standard risk indication for transplant of metabolic diseases that encompassed Wilson's, Haemochromatosis and Amyloid, 5 year survival fell by 10%, to 76%. Similarly, 5 year DCD graft survival fell a further 10% to 64%, in the high risk recipient group of HCV, HCC, ALD, cryptogenic and Budd Chiari (Figure 1). When MELD was used to define DCD risk, recipients with a MELD $\leqslant$ 25 were found to have a graft survival of 76% at 5 years, with survival falling a further 20% in higher MELD recipients. The use of DCD in reTPL was uncommon, accounting for 3.5% of DCD usage and graft survival was poorer (65% *vs* 78% at 5 years reTPL v primary transplant). Additionally, a donor hepatectomy time over one hour resulted in poor graft survival of 32% at 5 years. Allocating points, to the risk associated with each of these variables produced the DCD-RI score (Table 2).

A DCD-RI score over 5 was high risk for early graft loss, and predictive for poor long term survival of 34% at 5 years (Figure 5). In order to minimize DCD graft loss, the ideal is to aim for a DCD-RI score less than 5, which can be achieved either by minimizing the risk from the donor by selecting/aiming for a short WIT < 25 min, short CIT < 10 h, dHepT < 60 min, or by negating the DCD risk of the donor by selecting a low risk recipient, *i.e.,* MELD < 25, not a reTPL or belonging to the low risk primary indication group for DCD transplant. Internal validation of the DCD-RI on an earlier cohort supported the validity of the developed DCD-RI score by its ability to accurately predict graft survival for that cohort. Additionally, comparison, of the DCD-RI score showed it to out perform other predictive scoring systems such as the DRI and MELD.

Calculating the DCD-RI score helps to provide a framework to rationalize some of the risks involved in DCD liver transplantation. However, there are limitations to the data that were used to build the DCD-RI scoring system. The transplanted DCD livers whose data was used to design the DCD-RI score were already highly selected[10], automatically introducing bias into the study. By preselecting good quality DCD livers[21] as reflected by young donor age and short ischemic times (cold and warm), the majority (94.6%) of DCD transplants used to develop the DCD-RI belong to the low and standard DCD-RI score risk classes, while high risk DCD-RI transplants (DCD-RI score > 5) were rare in the programme. But by being stringent in DCD selection good outcomes can be achieved, comparable to DBD in both the short and long term[22].

Another factor that is well recognized to be a determinant of outcome in DCD transplantation but was not included in the DCD-RI was steatosis. The main reason for exclusion was assessment of liver steatosis by the surgeon is highly subjective[23] and histological assessment of the donor liver pre-perfusion is not routinely performed in transplant, and is in itself, a subjective assessment. Institutionally, the steatotic (> 30%) DCD liver is not used which may explain why donor BMI and donor liver weight, both surrogate markers of liver steatosis, were not found to have any bearing on graft survival. Neither, donor or recipient age, were included in the DCD-RI model as they were not found to be determinants of outcome in the data analyzed. This again reflects institutional practice, which is not to use donor/recipient age on its own, as a reason for DCD non consideration. Therefore, the developed DCD-RI has been able to combine adult and pediatric data. However, donor age has been identified in other series as a risk factor for graft failure[18] but older donors can be a valuable source of organs, and the risk from age can be balanced by reducing the risk from an alternative donor or recipient factor(s), *e.g.*, CIT[24,25].

Other predictive models for outcome after liver transplantation have been explored but they all, as does the DCD-RI, have various limitations. The donor recipient index (DRI) considers only donor factors[26]. While, the MELD score, that is the foundation of liver allocation on transplant waiting lists[27-29] is a poor predictor of outcome after transplant[30]. A number of other complex models detailing interactions between donor and recipient risk profiles have been developed to predict graft and patient survival after liver transplantation[30-39]. But none consider DCD in isolation and it is well recognized, that the DCD liver is a different type of graft in comparison to DBD, and DBD predictors of outcome have not been found to be applicable to DCD[40].

Only one other group has tried to design a DCD

prognostic scoring system, admittedly with smaller numbers (n = 81), in adults only and is yet to be validated[41]. However, they found similar variables to that of the present DCD-RI to be important predictors of DCD graft survival, such as primary indication for transplant, retransplantation, donor warm ischemic time and cold ischemic time (< 6 h). But with their DCD data, unlike the present data, they found recipient BMI (> 30) and donor HBV core antibody status influenced DCD graft outcomes. They did not consider donor hepatectomy time.

In conclusion, the developed DCD-RI score helps to rationalize and balance the risk between the donor and the recipient in DCD liver transplantation, in order to achieve good graft survival. To determine the true utility of the system it will need to be prospectively validated in other large volume DCD programmes.

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COMMENTS

Background

In Liver Transplant programmes in the West, there is a reliance on cadaveric donors over living related. The availability of cadaveric organs is insufficient to meet transplant need. Therefore, more marginal cadaveric organs have to be used, one such organ, is the donor after cardiac death (DCD) liver. However, this is a high risk organ and difficult to use with good results, most of which depends on subjective experience driven knowledge. The aim of this work was to create a scoring system using pre transplant objective data points from the donor and recipient to rationalize this risk.

Research frontiers

There is a lack of data on how to achieve the balance of risk between objective clinical variables from the DCD liver and selected recipient to produce good results. The present work aims to address this lack of information.

Innovations and breakthroughs

The DCD Risk Index (DCD-RI) score that was developed using objective variables from the donor and recipient was able to predict graft loss and DCD-RI class predicted graft survival.

Applications

The DCD-RI is a tool that can help in decision making on whether to use a given DCD liver in the selected recipient to produce good results.

Peer-review

Both internal and external was performed in order for this manuscript to be accepted for publication.

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CASE REPORT

Tuberculosis in kidney transplant recipients: A case series

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Abstract

Solid organ transplant recipients have an elevated risk of tuberculosis (TB) with high mortality. Data about TB in this population in the United States is sparse. We present four cases of active tuberculosis in kidney transplant recipients at our center. All patients had possible TB exposure prior to transplant and all were diagnosed with active TB within the first year of transplant. Disseminated TB was seen in half of the patients with extra-pulmonary TB being more common affecting lymph nodes, pericardium, and the kidney allograft. Delay in diagnosis from onset of symptoms ranged from fifteen days to two months. Two patients died from TB. TB is a largely preventable and curable disease. However, challenges remain in the diagnosis due to most recipients presenting with atypical symptoms. Physicians should maintain a high degree of suspicion for TB to promptly diagnose and treat post-transplant thereby minimizing complications. A review of the literature including the epidemiology, pathogenesis, clinical presentation, diagnosis and treatment options are discussed.

Key words: Mycobacterium tuberculosis; Kidney transplant; Disseminated disease; Tuberculosis

Core tip: Tuberculosis is a largely preventable and curable disease that should be suspected in all solid organ transplant recipients who present with unexplained fevers, pulmonary or extra-pulmonary symptoms. This case report describes the varied presentations of tuberculosis in kidney transplant recipients and provides the most recent recommendations regarding diagnosis and treatment.

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INTRODUCTION

The overall incidence and prevalence of mycobacterium tuberculosis (TB) in solid organ transplant recipients is not well defined. The rates of TB in this population are mostly based on data available from individual study cohorts reported in the literature. In the western world, TB is a rare opportunistic infection with significant morbidity and mortality. Clinical presentation in immunocompromised individuals, including transplant recipients is often atypical and diverse. This leads to delay in the diagnosis and advanced disease at the time of diagnosis. In addition, inadequate host response in this setting poses a treatment challenge. The higher toxicity of treatment and concurrent use of immunosuppressive medications with drug interactions further generate complexity in management. We describe four cases of active TB in our kidney transplant recipients and explore the epidemiology, clinical presentation, management and outcomes of TB disease in this population.

CASE REPORT

Case 1

A 63-year-old Vietnamese male with end stage renal disease due to IgA nephropathy received an expanded criteria deceased donor kidney transplant (DDKT) in 2012 (5 antigen mismatch, 5% panel reactive antibody, PRA). He received induction with alemtuzumab and solumedrol and was maintained on tacrolimus and mycophenolate mofetil. There were no surgical complications or episodes of acute rejection in the post-transplant period. Allograft function stabilized with a serum creatinine (Cr) of 1.8 mg/dL. His past medical history was notable for incarceration in Vietnam, prior hepatitis B exposure with protective anti-Hepatitis B surface antibody, positive tuberculin skin test (TST) and a non-calcified nodule on chest X-ray (CXR). He had been in the United States for twenty years prior to his transplant. He did not receive isoniazid (INH) prophylaxis before undergoing kidney transplant. At one-year post-transplant, he was admitted with fever, palpitations and 3 cm non-tender submental lymph node. Labs were notable for acute kidney injury (AKI) with Cr of 3 mg/dL and urinary retention that resolved with urinary catheter placement and treatment for an enlarged prostate. CXR revealed bilateral pleural effusions and a large pericardial effusion. Fine needle aspiration of the lymph node and pericardial fluid grew Mycobacterium tuberculosis (MTB). He received anti-tubercular therapy (ATT) with 2 mo of Rifampin, INH, Pyrazinamide and Ethambutol (RIPE) and 4.5 mo of INH and Rifampin (IR). His treatment course was complicated by transaminitis with reactivation of hepatitis B leading to end stage liver disease. He was treated with tenofovir with resolution of transaminitis. Patient completed 6.5 mo of ATT and has been cured of TB. His kidney transplant failed three years later due to BK nephropathy, and he was initiated on hemodialysis.

Case 2

A 67-year-old Caucasian male, Vietnam War veteran with ESRD presumed secondary to hypertension received a DDKT in 2013 (0 antigen mismatch, PRA 36%). He received induction with alemtuzumab and solumedrol and was maintained on tacrolimus, mycophenolate mofetil, and prednisone. Pre-transplant CXR showed prior granulomatous disease. He was not tested for latent TB infection (LTBI). Two months after transplant, he was admitted with fever and progressive shortness of breath. CXR revealed a miliary pattern of infiltrates. He developed acute respiratory failure and septic shock requiring intubation and multiple vasopressors. The day after admission, sputum samples returned positive for acid-fast bacilli (AFB), and later grew MTB. Clinical course was complicated by development of presumed macrophage activation syndrome (MAS). He received neupogen for pancytopenia but bone marrow biopsy could not be obtained due to agitation. He did not receive intravenous steroids or chemotherapy for MAS. Patient died within three days of admission.

Case 3

A 38-year-old Indonesian woman living in United States for ten years with ESRD due to IgA nephropathy on hemodialysis for 10 years received a DDKT in 2015 (6 antigen mismatch, PRA 0%). She received induction with alemtuzumab and solumedrol and was maintained on tacrolimus, mycophenolate mofetil, and prednisone. There were no surgical complications or episodes of acute rejection in the post-transplant period. Allograft function was excellent with serum Cr of 1.0 mg/dL. Pre-transplant work up was notable for positive TST with normal CXR. She was started on INH immediately after transplant and received nine months of therapy for LTBI. One month after completing INH therapy, she was admitted with persistent fevers, night sweats and acute kidney injury, serum Cr of 2 mg/dL. Fever work up showed adenovirus in the blood and urine. There was increased flurodeoxyglucose uptake in the kidney allograft on positron emission tomography scan. Biopsy of the kidney transplant showed necrotizing granulomatous interstitial nephritis. Differential diagnosis of the granulomatous interstitial nephritis included renal transplant TB and adenovirus infection. Renal pathology changes were not consistent with adenovirus infection. AFB smear and cultures were negative in the urine and renal biopsy specimens. Due to persistent fevers, worsening renal function and clinical suspicion for TB, she was started on RIPE and Moxifloxacin. Moxifloxacin was added as a fifth agent due to concern for INH resistance given she was treated with INH monotherapy for LTBI. Fevers, night sweats and AKI resolved on treatment without addition of cidofovir, which supported the diagnosis of renal transplant TB. Her IS was modified with discontinuation of MMF. She is currently maintained on tacrolimus and prednisone. She completed 6 mo of ATT and is cured of TB. Renal allograft function is stable

with Cr of 1.3 mg/dL.

Case 4

A 67-year-old Caucasian male with ESRD, secondary to diabetes mellitus on hemodialysis for 2 years received a DDKT in 2015 (4 antigen mismatch, PRA 0%, A2 to B kidney). He received induction with alemtuzumab and solumedrol and was maintained on tacrolimus, mycophenolate mofetil, and prednisone. His pre-transplant CXR showed calcified lung nodules, and he had a negative interferon gamma release assay (quantiferon gold). He presented two and a half months' post-transplant with two weeks of intermittent fever, malaise, progressive dyspnea and lower extremity swelling. He was diagnosed with bilateral lower extremity deep vein thrombus and pulmonary embolism for which anticoagulation was initiated. Due to intermittent fevers, computed tomography (CT) of the chest was done that showed a few scattered sub centimeter non-calcified pulmonary nodules and a 2 cm right paratracheal lymph node concerning for granulomatous disease. Fungal testing including serum galactomannan, serum cryptococcal antigen, beta-D-glucan levels and urine histoplasma antigen, was negative. Bronchoscopy was performed with AFB stain positive in the bronchoalveolar lavage (BAL). AFB and non-necrotizing granulomas were seen on trans-bronchial lung biopsy. MTB complex polymerase chain reaction (PCR) was positive in both the BAL and blood, and cultures from both grew MTB. Sputum cultures later grew pan susceptible MTB. He was discharged on a four-drug regimen with RIPE. Two weeks later, he was readmitted with recurrence of fever, altered mental status and partial loss of vision. Repeat CT of the chest showed worsening bilateral pulmonary infiltrates.

Moxifloxacin was added to his regimen. ATT drug levels were obtained and found to be therapeutic. Sputum, urine and blood cultures returned negative for AFB. Neurology work up including magnetic resonance imaging of the brain and lumbar puncture was negative. Patient developed AKI with serum Cr of 3 mg/dL. Ethambutol dose was decreased from 1600 mg daily to 1600 mg every 36 h and pyrazinamide dose was lowered from 2000 mg daily to 2000 mg thrice weekly. Ethambutol was subsequently discontinued due to worsening visual changes and amikacin was added to the treatment regimen of isoniazid, rifampin, pyrazinamide and moxifloxacin. His IS was ultimately tapered to prednisone alone due to worsening of TB with persistent fever and progressive pulmonary infiltrates. Renal allograft function continued to decline likely due to tapering off IS and aminoglycoside toxicity ultimately leading to allograft failure. He was started on hemodialysis 4 mo after initiation of ATT and died three months later.

DISCUSSION

Epidemiology

Even before MTB was discovered, Laennec described the diseased lung cavities on autopsies. Historically this was referred to as "consumption" owing to significant weight loss and finally death that consumed patients. In 1839, Johann Schonle coined the term tuberculosis from the Latin word "tuberculum" which means small pimple or a bump. The bacillus was identified by Robert Koch as Mycobacterium tuberculosis on March 24, 1882 which is commemorated as World TB day.

The global TB incidence and prevalence has been declining per the most recent WHO Global TB report[1]. The incidence of TB globally is 18% lower in 2014 as compared to 2000 and TB prevalence is 42% lower as compared to 1990. TB mortality has also fallen 47% since 1990. The incidence rate is highest in South East Asia and the Western Pacific and lowest in Western Europe, Canada, United States, Australia and New Zealand. The CDC Morbidity and Mortality Report in early 2016 shows leveling of TB incidence in the United States at 3 cases/100000 persons in 2013-2015, after two decades of annual decline[2]. Approximately 70% of the cases are in foreign-born individuals, with Mexico, the Philippines, India, Vietnam and China accounting for the top five countries of origin. In our case series, two out of four were from Southeast Asia which is considered to be endemic for TB. Among those born in the United States, native Hawaiians/other Pacific Islanders have the highest incidence followed by American Indians and Alaskan Natives. Almost half of all reported TB cases in the United States are reported from California, Florida, New York and Texas. The TB incidence in foreign born individuals has been steadily declining compared to stabilization of TB incidence among those born in the United States, pointing to TB transmission in the United States. This has been confirmed by molecular genotyping. Risk factors for TB outbreaks include substance abuse, incarceration and homelessness.

Data regarding the prevalence and incidence of TB in solid organ transplant recipients is sparse. Prevalence of active TB is estimated to be 1.2%-6.4% in developed countries and up to 15% in highly endemic areas[3]. A study in 1998 estimated a 0.35%-1.2% incidence in renal transplant recipients in the United States[4]. Risk in solid organ transplant recipients is estimated to be 20-74 times higher than the general population with a high mortality rate of up to 30%. Mortality of TB is higher in patients with disseminated disease, prior rejection and those who received anti-T cell antibody therapy[4]. Another study found higher mortality with graft rejection, steroid treatment and concomitant other opportunistic infection[3]. Diabetes mellitus and chronic liver disease have also been associated with greater mortality[5]. Our case series show a mortality of 50%. Half of our TB cases had disseminated disease. All four patients received anti-T cell antibody therapy and three were on steroids. Half of our patients had diabetes mellitus. Baseline characteristics of our patients are listed in Table 1.

Over 50% of renal transplant recipients develop TB within the first year of transplant[4]. TB develops earlier

Table 1 Baseline characteristics of patients

	Patient 1	Patient 2	Patient 3	Patient 4
Age (yr)	63	67	38	67
Ethnicity	Vietnamese	Caucasian	Indonesia	Caucasian
Sex	Male	Male	Female	Male
BMI (kg/m^2)	32	33	21	32
Prior TB exposure	Incarceration in Vietnam	Vietnam war veteran	Lived in Indonesia till age 25	None
PPD/IGRA	Positive	Not done	Positive	Negative
Pre-transplant CXR	Non-calcified lymph nodes	Prior granulomatous disease	Normal	Calcified lung nodules
Smoking	Yes	No	No	No
Diabetes mellitus	Yes	No	No	Yes
Hepatitis C	No	No	No	No
Chronic liver disease	Prior hepatitis B exposure	No	No	No
Pre-transplant INH prophylaxis	No	No	No	No

BMI: Body mass index; TB: Tuberculosis; PPD: Purified protein derivative; IGRA: Interferon gamma release assay; CXR: Chest X-ray; INH: Isoniazid.

in those with prior TB exposure[3]. Markers for prior infection include cellular response to TB specific antigens (positive TST or interferon gamma release assay, IGRA) or sequelae of granulomatous infection on CXR. Older patients are more likely to have reactivation following transplantation than younger patients, particularly in the developed world. All of our cases had prior TB exposure and developed TB early after transplant, half developed disease within the first 3 mo following transplant.

Factors predisposing to TB both in the general population and transplant recipient include country of origin, history of untreated latent TB infection, cigarette smoking, body mass index < 18.5, diabetes mellitus, chronic kidney disease, chronic liver disease, lupus, human immunodeficiency virus, silicosis, gastrectomy, jejuno-ileal bypass, as well as social risk factors (homelessness, incarceration, alcoholism and known TB contact)[6,7]. The main predisposing factor in our center's experience was residence from or previous travel to an endemic region (Table 2).

Pathogenesis

TB is usually acquired *via* inhalation of bacilli into the lungs. Progression to clinical disease depends on the infecting dose and virulence of the Mycobacteria as well as the development of host cell mediated immunity. The most common reason for post-transplant TB is reactivation of previous infection. In patients with prior exposure, the risk is generally inversely related to the time from acquisition to transplantation. Rarely, TB can be donor-derived and transmitted through the transplanted organ. TB can be acquired post-transplant, more commonly in TB endemic countries, or nosocomial as part of outbreaks in renal transplant units[8].

Clinical presentation

The clinical presentation of TB in transplant recipients differs from the general population in that symptoms are more unusual and varied, often leading to a delay in diagnosis and poor outcomes. Fever is seen more commonly, and approximately 30%-50% of TB after transplant is extra-pulmonary or disseminated[4,7]. Disseminated disease is defined as involvement of two or more non-contiguous organs with positive TB cultures, with or without granulomas[4].

CXR's in post-transplant TB show diffuse pulmonary infiltrates rather than cavitary lesions which are more commonly seen in the general population[7]. In our case series, fever was present in all four patients. Cervical lymphadenopathy was seen in one patient. Disseminated TB was seen in two of the four patients with extra-pulmonary involvement of lymph nodes, pericardium and the renal allograft. Two patients had pulmonary TB and one of them had disseminated disease. Only one presented with cough. Patients with pulmonary involvement showed military pattern and bilateral diffuse pulmonary nodules on CXR.

Diagnosis and pre-transplant screening

Diagnosis of latent TB is an indirect measure of possible infection by detection of a cellular response to MTB specific antigens in the absence of symptoms. The two types of tests are *in-vivo*: Tuberculin skin test (TST) done by intradermal injection of purified protein derivative (PPD); and *ex-vivo*: IGRA (Quantiferon gold test or T-spot TB test). PPD is a glycerol extract of the tubercle bacillus and is not species specific. Induration of 5 mm or more is considered to be positive in transplant candidates. If the first test is negative, a follow-up second test is recommended two weeks later. This leads to a "booster effect" due to amnestic recall of immunity and can identify an additional 10% of cases[9]. Limitations of PPD testing include a higher rate of false negatives in the immunocompromised host, confounding by non-tubercular mycobacteria and prior BCG vaccination, and need for trained staff and a second visit for interpretation of the test by a qualified provider. IGRA utilizes sensitized T cells that release interferon-gamma. The advantages of IGRA over PPD include improved specificity due to MTB specific antigens that do not cross-react with BCG and the use of positive and negative controls that may differentiate true negatives from those that result from anergy or overt immunosuppression[10]. Performance of IGRA is better in low prevalence countries as compared to endemic

Table 2 Post-transplant patient characteristics and outcomes

	Patient 1	Patient 2	Patient 3	Patient 4
INH prophylaxis	No	No	Yes	No
T cell depleting antibody	Yes	Yes	Yes	Yes
Immunosuppressive	Tacrolimus, MMF	Tacrolimus, MMF	Tacrolimus, MMF	Tacrolimus, MMF
Corticosteroid	No	Yes	Yes	Yes
Acute rejection (6 mo prior to TB diagnosis)	No	No	No	No
Clinical features	Fever, palpitations, cervical LN	Fever, shortness of breath, cough	Fever, acute kidney injury	Fever, shortness of breath, leg swelling
TB site	Disseminated	Pulmonary	Extra-pulmonary	Disseminated
Time to symptom onset (mo)	11.5	2	9	2
Time to diagnosis, post-transplant (mo)	12	3	11	3
Treatment regimen	RIPE	None	RIPE-M	RIPE-M, Amikacin
Treatment duration (mo)	6.5	N/A	6	7
Adverse drug reaction	Hepatotoxicity	N/A	None	Neurologica,vision loss
Other complication	HBV reactivation, acute liver injury	Septic shock, MAS	None	VTE, IRIS, allograft failure
Outcome	Cured	Death	Cured	Death

INH: Isoniazid; MMF: Mycophenolate mofetil; TB: Tuberculosis; LN: Lymphadenopathy; RIPE: Rifampin, isoniazid, pyrazinamide, ethambutol; RIPE-M: Rifampin, isoniazid, pyrazinamide, ethambutol, moxifloxacin; HBV: Hepatitis B virus; MAS: Macrophage activation syndrome; VTE: Venous thromboembolism; IRIS: Immune reconstitution inflammatory syndrome.

areas[11]. Both these tests cannot differentiate between latent TB and active TB. ESRD and immunosuppressant use are responsible for a higher rate of false negative or equivocal results of immune based T-cell assays. Uremia is associated with impaired co-stimulatory function of the antigen-specific T-cells leading to a defect in T-cell function. One of our transplant recipients had a negative IGRA in the presence of calcified nodules on chest imaging.

Immunosuppressants such as T-cell depleting antibodies, corticosteroids and calcineurin inhibitors cause a reduction in the number of T-cells, affect their interaction with antigen-presenting cells and impair cytokine induction[12].

Diagnosis of TB in transplant recipients is often delayed. In our case series, delay in diagnosis from onset of symptoms ranged between fifteen days and two months. Diagnosis of active TB is made by demonstration of AFB on smear microscopy and isolation of mycobacteria in culture of the body fluid. AFB blood cultures should be done if there is a suspicion for disseminated TB. For pulmonary TB, three samples of sputum are sent 8-24 h apart with at least one being an early morning sample. Sputum induction with aerosolized hypertonic saline can be employed for patients who are unable to expectorate. Invasive diagnostic tests such as bronchoscopy with bronchoalveolar lavage may be necessary for diagnosis. Sensitivity and specificity of sputum AFB smear microscopy is 45%-80% and 50%-80%, respectively[13].

Sensitivity and specificity of sputum culture is 80% and 98%, respectively[14,15]. Cultures need to be incubated for 6-8 wk to isolate MTB. Drug susceptibility testing should be done on all positive MTB cultures. Nucleic acid amplification (NAA) assays are available for rapid diagnosis of TB. These tests can be done from cultures or direct tissue samples. The Centers for Disease Control (CDC) recommends sending the first sputum sample for NAA testing. These assays can detect target specific MTB complex RNA/DNA sequences with nucleic acid probes in 24-48 h. Xpert MTB/Rif test is an automated NAA test that detects rifampin resistance simultaneously in two hours. Rifampin resistance is a marker of multi-drug resistant (MDR) TB. Sensitivity and specificity of NAA tests in AFB smear positive respiratory secretions is over 95% and is not affected by non-tuberculous *Mycobacteria* (NTM) or immunosuppression. They have lower sensitivity, 75%-85% in smear negative sputum[16-18]. These tests should be performed within the first few days of ATT and a negative NAA test does not exclude TB. Cultures are still required for species identification and for drug susceptibility testing. NAA assays do not perform as well for other clinical specimens and the overall evidence regarding their use in transplant patients is lacking at this time. Tissue biopsy of the involved organ and/or fluid for histopathology evaluation, AFB smear and culture should be obtained in suspected extra-pulmonary TB.

In our case series, we diagnosed TB disease if any of the following criteria were met: (1) isolation of MTB in culture of sputum, blood or any body fluid, with or without detection of AFB on smear; (2) clinical response to ATT in a patient with fever of unknown origin or compatible clinical syndrome with radiographic and histopathological features suggestive of TB, including tissue sample with granulomas; and (3) presence of MTB DNA using PCR.

Pre-transplant screening of donor and recipient for TB infection should be rigorous given the high risk of TB in the transplant setting and significant associated mortality. In transplant candidates and living donors, thorough history taking and comprehensive physical examination should be performed with a special focus on the medical and social risk factors for TB mentioned earlier. History of TB exposure is most essential and one should inquire about residence and travel history to endemic areas, contact with a known active TB case, and prior TST testing results. In patients with a history of prior LTBI or TB, details regarding treatment regimen and duration are essential, and active TB in these individuals should

be excluded. These patients may need additional testing and consultation with a transplant infectious disease specialist. Donors with active TB within 2 years have higher risk of relapse and transmission *via* the allograft[6]. Patients without prior history of known LTBI or TB disease should undergo testing for LTBI with a PPD test or IGRA. If the first PPD test is negative, a second skin test is recommended for booster effect as discussed earlier. A CXR is part of routine preoperative screening and should be evaluated for evidence of prior granulomatous disease. Patients with positive PPD or IGRA should be treated for LTBI prior to transplantation, whenever possible, after exclusion of active TB. Individuals with low risk of TB based on history and negative testing are cleared for transplantation. In high risk patients with negative TST/IGRA, indeterminate IGRA or chest imaging suggestive of prior granulomatous disease, it is recommended to treat with INH for presumed LTBI, prior to transplantation. Active TB needs to be ruled out by appropriate smears, cultures and molecular testing before treatment for latent TB is initiated. In high-risk patients, urine for AFB and renal imaging should also be performed to rule out genitourinary TB[19]. In our case series, two patients had known LTBI by PPD/IGRA but did not receive INH prophylaxis prior to the transplant. One of the patients received INH prophylaxis immediately post-transplant. One patient was not tested for LTBI, but was high risk based on prior exposure history and a CXR with old granulomatous changes. Interestingly, one recipient tested negative by IGRA and was low clinical risk. He had calcified nodules on imaging and later developed TB disease.

Pre-transplant evaluation is challenging in deceased donors given the limited history available. Efforts should be made to obtain a history regarding prior TB exposure, TB disease and treatment from family and healthcare givers. The evaluation is similar to living donors as above, prior to accepting the organ. In donors with a history of TB and reliable information about completed ATT, appropriate smears, cultures and molecular testing should be done to rule out active disease. In deceased donors with a history of TB disease and insufficient information about treatment or positive testing, it is recommended to reject the donor except in urgent transplants. In this scenario, recipients should be treated for active TB after informed consent with close monitoring under the guidance of an infectious disease specialist[6,8].

Management

Direct evidence regarding management of transplant recipients for prevention and treatment of latent and active TB infection is lacking. Their care is largely based on expert opinion and extrapolation from studies in immune-competent and other immunocompromised populations. Indications for treatment of LTBI in recipient candidates include a positive TST/IGRA as well as those with a negative TST/IGRA or indeterminate IGRA with risk factors: Radiographic evidence of prior TB in the absence of treatment, donor with recent TB exposure, positive TST or radiographic signs, or close prolonged contact with an active TB case[8]. Before treatment of LTBI, active TB needs to be excluded. One recipient in our case series with a positive PPD received INH prophylaxis soon after transplant for 9 mo. However, a month after finishing INH, she developed renal allograft TB. This patient was asymptomatic, but cultures were not obtained prior to initiation of prophylaxis. The other explanations for the development of active TB include possible low levels of INH due to concomitant steroids and inadequate host response in the setting of immunosuppressant use post-transplant. Treatment regimens for LTBI include INH 5 mg/kg daily (maximum dose 300 mg/d) for 9 mo with pyridoxine 25-50 mg daily to prevent neurotoxicity. INH 15 mg/kg twice weekly (maximum dose 900 mg/d) with pyridoxine, given as directly observed therapy has also been proposed. Rifampin 10 mg/kg daily (maximum dose 600 mg/d) for four months may be used prior to transplant but should be avoided if possible after transplant due to drug interaction with the immunosuppressant medications. Combination of pyrazinamide and rifampin daily for 2 mo is not recommended due to the high risk of hepatotoxicity in the transplant population. A shorter regimen of weekly INH and rifapentine for 12 wk, as directly observed therapy, to treat immune competent individuals is not recommended in renal transplant candidates[8]. Compliance with LTBI treatment is poor as seen in a North American study where only half of the patients initiated on therapy finished the complete course of treatment[6]. If treatment is interrupted for more than two months, patients should be excluded again for active TB[12]. Adverse effects are more common in solid organ transplant recipients with hepatotoxicity seen in 37% of kidney recipients and up to 50% in liver transplant recipients[8,20]. Monitoring should involve monthly physician examination and bi-monthly blood levels of liver function tests.

Medications will need to be discontinued or dose adjusted if liver function tests are more than three times the upper limit of normal with symptoms/signs, or more than five times the upper limit of normal without symptoms[12].

Treatment of active drug susceptible TB usually involves two months of an initial phase therapy with INH, rifampin/rifabutin, pyrazinamide, +/- ethambutol, followed by a continuation phase therapy of four months of INH and rifampin, with a total duration for six months. Cavitary TB, with positive sputum culture after two months of intensive phase therapy, is treated for nine months' duration with prolongation of continuation phase therapy. Bone and joint disease as well as severe disseminated disease are treated for a total of six to nine months. Central nervous system disease warrants treatment duration of nine to twelve months[8]. Since the majority of transplant recipients present with severe disseminated TB, 9 mo or longer duration of treatment may be preferred in the presence of response to ATT. Risk of recurrence was found to be lower when treatment is extended to beyond 12 mo[12]. Longer course of therapy is required if second line drugs are used

due to adverse effects or in cases of drug resistant TB.

MDR and extensively drug resistant TB fortunately has been rarely reported in solid organ transplant recipients. This should be treated according to drug susceptibility testing with at least four active drugs. The World Health Organization (WHO) suggests a total treatment duration of 18 mo after culture conversion. Adjunctive surgery may be required in some patients[12].

In the United States, patients with pulmonary TB have sputum cultures obtained monthly until two consecutive cultures are negative, and at two months of intensive phase therapy to further guide treatment. If the sputum culture at two months of treatment is positive, WHO recommends sputum smear microscopy at the end of the third month and if positive, sputum culture and drug susceptibility testing. Drug susceptibility testing should also be done if a patient develops positive cultures after a period of negative cultures. European guidelines in transplant recipients recommend sputum smear and culture at a minimum of two months and four months of treatment, at the end of ATT, and on two further occasions until the end of 12 mo[12]. Extra-pulmonary TB in general is followed clinically. Patients should have baseline laboratory data including a comprehensive metabolic panel, complete blood counts, and uric acid levels. They should be monitored and managed for hepatotoxicity as described above. Baseline and monthly visual acuity and red-green discrimination testing should be done with ethambutol use.

If one suspects pulmonary TB, the patient should be isolated in a negative pressure room until active TB is excluded. Pulmonary TB patients should be isolated for at least two weeks with clinical improvement on therapy and until three consecutive negative sputum smears are obtained. In immunocompetent patients, rapid testing with Xpert MTB/Rif has been used in conjunction for decisions regarding discontinuation of TB isolation. However, this cannot be recommended in the transplant population at this time.

Drug interactions

Patients need to be monitored closely for drug interactions with immunosuppressive medications used in solid organ transplant given the increased risk of rejection. Rifampin is used in treatment of TB due to its potent MTB sterilizing action. Rifampin is a strong inducer of CYTP3A4 leading to increased metabolism of calcineurin inhibitors, mammalian target of rapamycin (mTOR) inhibitors, mycophenolate mofetil and corticosteroids. Rifabutin is a less potent cytochrome inducer. Drug levels need to be monitored closely at initiation of TB therapy, after discontinuation of rifampin or rifabutin, or with any adjustment of immunesuppressant dosing[8]. Spanish guidelines recommend rifamycin free regimens for treatment, except for disseminated TB and INH resistant TB[19]. We prefer rifamycin based regimens for treatment of TB in our renal transplant recipients. Other drug interactions to consider include the following: INH may increase corticosteroid levels and its adverse effects, streptomycin with cyclosporine and sirolimus may cause additive nephrotoxicity, fluoroquinolones can further increase risk of tendon rupture with concomitant corticosteroids, and corticosteroids may decrease INH levels[12].

Complications

Complications of TB besides primary organ involvement include septic shock, venous thromboembolism (VTE), immune reconstitution inflammatory syndrome (IRIS) and macrophage activation syndrome (MAS) or hemophagocytic syndrome[21,22]. Septic shock with TB is associated with high mortality[23]. Pulmonary and extra-pulmonary TB both predispose to VTE with the risk being much higher than other hospitalized patients, in general[24,25]. IRIS is recognized by the paradoxical symptom worsening of fever, cough, enlarging lymph nodes or worsening of findings on imaging after initiation of treatment. This is seen primarily in the first few months of initiation of therapy. MAS is rare and has high mortality. It manifests as fever, hepatosplenomegaly, pancytopenia and liver abnormalities. Diagnosis is usually made by bone marrow biopsy showing infiltration of non-malignant macrophage phagocytizing red blood cells[12,21]. In our case series, one patient presented with septic shock and presumed MAS succumbing to his illness. The other patient presented with VTE and developed IRIS two months after initiation of ATT.

In conclusion, TB remains a challenging opportunistic infection in the solid organ transplant population. Efforts should be made to prevent active TB *via* recognition and treatment of LTBI in potential donors and transplant candidates, ideally prior to transplantation. Current tests for LTBI (PPD and IGRA) can be falsely negative in patients with ESRD and those on immunosuppressive medications. IGRA has not been evaluated for use in deceased donors. There is a need for better diagnostics for LTBI. Exclusion of active TB is of paramount interest prior to LBTI therapy by culture, smear, imaging and molecular testing as needed. Given the changes in the allocation system, older and longer dialysis vintage recipients are being transplanted, increasing the risk of active TB. Due to the organ shortage with more high risk donors being utilized, the risk for donor derived TB might increase as well. More widespread use of rapid NAA assays and line probe assays is needed to screen high-risk TB donors, and for diagnosis of TB in recipients. As disseminated and extra-pulmonary disease are more common in transplant recipients, studies are needed to assess the performance of NAA assays in body fluids, other than sputum, in this population. Given diagnostic limitations, physicians need to maintain a high clinical suspicion for TB post transplantation in order to initiate early treatment and decrease morbidity and mortality. Studies are needed to investigate the efficacy of shorter treatment regimens given the interactions with immunosuppressive medications and significant adverse effects. Lastly, public health efforts are needed both at the

global and domestic level to minimize this disease.

COMMENTS

Case characteristics

Four kidney transplant recipients, aged 38-67 years, presenting with fever within one year of kidney transplantation.

Clinical diagnosis

Lymphadenopathy, pleural effusion, pericardial effusion, acute respiratory failure, septic shock, acute kidney injury, bilateral lower extremity deep venous thrombosis and pulmonary embolism.

Differential diagnosis

Bacterial infections, fungal infections such as histoplasmosis, cryptococcosis, interstitial nephritis due to adenovirus infection, post-transplant lymphoproliferative disorder.

Laboratory diagnosis

Demonstration of acid-fast bacilli in sputum and bronchoalveolar lavage. Mycobacterium tuberculosis grew in cultures from sputum, blood, lymph node aspirate and pericardial fluid. Positive Mycobacterium tuberculosis PCR in blood and bronchoalveolar lavage.

Imaging diagnosis

Radiological features included calcified/non-calcified lung nodules, diffuse lung infiltrates, pleural effusion, lymphadenopathy, pulmonary embolism and increased flurodeoxyglucose uptake in the kidney allograft on positron emission tomography scan.

Pathological diagnosis

Necrotizing and non-necrotizing granulomas seen on kidney allograft and transbronchial lung biopsies respectively. Demonstration of acid-fast bacilli on lung biopsy.

Treatment

Two months of Rifampin, Isoniazid, Ethambutol and Pyrazinamide followed by 4 mo of Rifampin and Isoniazid. Second-line drugs moxifloxacin and amikacin were used in selected cases.

Related reports

Tuberculosis in solid organ transplant recipients is rare in the developed countries. A study in 1998 estimated 0.35%-1.2% incidence in the United States.

Term explanation

Tuberculosis is a rare opportunistic infection caused by acid fast bacillus Mycobacterium tuberculosis that was identified by Robert Koch in 1884.

Experiences and lessons

Tuberculosis should be considered in solid organ transplant recipients presenting with unexplained fever to avoid delayed or missed diagnosis. TB carries high morbidity and mortality. Transplant recipients should have comprehensive screening for risk factors for TB along with testing for latent TB. Active TB needs to be ruled out prior to the treatment of latent TB. Ideally patients should be treated for latent TB prior to transplant due to drug interactions and suboptimal response to therapy in the setting of immunosuppression.

Peer-review

The data across the different trials is reviewed well. The benefits and adverse effects are clearly illustrated and summarized.

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