# UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

**Faculdade de Medicina**

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# ESTUDO LONGITUDINAL DE CURTO PRAZO DA REPRODUTIBILIDADE E DA RESPOSTA QUANTITATIVA DO RNA MENSAGEIRO ÀS MANOBRAS IMUNOSSUPRESSORAS PARA O TRATAMENTO DA REJEIÇÃO AGUDA DE TRANSPLANTES RENAIS. AVALIAÇÃO POR REAÇÃO EM CADEIA DA POLIMERASE EM TEMPO REAL EM CÉLULAS SANGÜÍNEAS.


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# Sumário

# Lista de Abreviaturas

**DRC –** Doença Renal Crônica

**DGF –** *Delayed Graft Function* - Disfunção Inicial do Enxerto

**HLA –** *Human Leucocyte Antigens* – Antígenos Leucocitários Humanos

**RA –** Rejeição Aguda

**NIC –** Nefrotoxicidade por Inibidores de Calcineurina

**NTA –** Necrose Tubular Aguda

**NCE –** Nefropatia Crônica do Enxerto

**IFTA –** *Interstitial Fibrosis and tubular atrophy* – Fibrose Intersticial e Atrofia Tubular

**RASC –** Rejeição Aguda Sub-clínica

**RNA –** Ácido Ribonucléico

**mRNA –** RNA mensageiro

**cDNA –** DNA complementar

**PCR –** *Polymerase Chain Reaction* – Reação em Cadeia da Polimerase

**ROC –** Receiver Operating Characteristic

**VPN –** Valor Preditivo Negativo

**VPP –** Valor Preditivo Positivo

**TCR –** Receptor de Célula T

**CPH –** Complexo Principal de histocompatibilidade

# Resumo

O objetivo do presente estudo foi avaliar longitudinalmente a acurácia diagnóstica da análise transcripcional de enxertos renais. Trinta e seis receptores de transplante renal foram avaliados pelo período de três meses pós-transplante. Amostras de sangue periférico para a análise dos genes da Perforina e TIM3 pela reação em cadeia da polimerase em tempo real foram coletadas nos dias 3, 4-6, 9-11, 14-16, 19-21, 24-26, 29-31, 44-46, 59-61 e 89-91 do pós-operatório. Biópsias por indicação e de vigilância foram realizadas para avaliar disfunção aguda do enxerto ou durante períodos de disfunção inicial do enxerto (DGF) respectivamente e foram interpretadas usando a classificação Banff de 2007. Treze pacientes apresentaram 16 episódios de rejeição aguda. Receptores com rejeição demonstraram níveis mais elevados de transcritos de mRNA do gene TIM3 em comparação com pacientes que não foram acometidos por episódios de rejeição (mediana da expressão gênica foi de 153,7% e 40,1% respectivamente, $p<0,01$). Da mesma maneira, a expressão gênica da Perforina foi elevada em pacientes com rejeição (mediana da expressão gênica foi de 136,6% e 46,5% respectivamente, $p<0,01$). Curvas ROC (receiver operating characteristic) mostraram uma área sob a curva para o gene do TIM3 de 0,752 (intervalo de confiança de 95%: 0,653 – 0,852). A expressão de mRNA de Perforina forneceu uma área sob a curva de 0,733 (com um intervalo de confiança de 95%: 0,580 – 0,809). A acurácia global da expressão gênica foi de 77% para o gene do TIM3 e de 66% para o gene da Perforina. A acurácia combinada dos dois genes alcançou

82%. Valores preditivos negativos foram de 96% nas três análises. A expressão gênica foi significativamente modulada por tratamento de rejeição, onde observou-se um decréscimo de 60% (para o gene do TIM3) e 48% (para o gene da Perforina) em comparação com amostras pré-rejeição. Concluindo, a abordagem longitudinal mostrou-se muito útil para exclusão do diagnóstico de rejeição e também ao determinar a eficácia de seu tratamento.

# Capítulo 1: Introdução

O transplante renal é o tratamento de escolha para pacientes com doença renal crônica (DRC) em estágio terminal (1). Entretanto, pacientes submetidos a transplante renal podem ser acometidos por episódios de rejeição aguda, considerada uma das principais complicações pós-transplante imediato, e que é caracterizada pela resposta do sistema imune do hospedeiro que reconhece e ataca antígenos presentes no órgão do doador (8). O diagnóstico de rejeição aguda em enxertos renais é baseado na identificação anatomopatológica de infiltrados linfo-monocitários em fragmentos corticais de tecido renal obtido através de biópsia de enxertos com disfunção (11).

O advento de drogas imunossupressoras mais potentes usadas em combinações e que atuam em diferentes sítios e mecanismos da resposta imune contribuíram para melhoras na sobrevida dos enxertos e na diminuição da incidência de casos de rejeição aguda (3,4). Contudo, as melhorias obtidas com o desenvolvimento destas drogas não resultaram em melhora significativa da sobrevida em longo prazo dos enxertos (5-7). Assim sendo, receptores de transplante renal requerem tratamento crônico com drogas imunossupressoras e estão sujeitos a rejeições, toxicidade das medicações e outras doenças que comprometem a sobrevida do enxerto.

A alorresponsividade crônica aos antígenos presentes nos transplantes provavelmente seja um dos principais causadores da rejeição crônica. Esta é caracterizada por fibrose do tecido renal e a seguir pela perda de função do órgão.

O método diagnóstico atualmente utilizado para o diagnóstico das rejeições é a biópsia renal, um método invasivo, caro, com problemas de representatividade, interpretação e associado a complicações (17-19).

Adicionalmente, as patologias associadas à perda de função pós-transplante renal, tais como, a fibrose intersticial e atrofia tubular (IFTA), a nefrotoxicidade por inibidores de calcineurina (NIC), a necrose tubular aguda (NTA) e as infecções virais, em especial pelo vírus polioma, são também diagnosticadas pela biópsia. A infecção pelo vírus polioma pode apresentar um quadro histológico muito semelhante ao da rejeição aguda, dificultando o diagnóstico diferencial. Muitas vezes é difícil diferenciar-se a rejeição crônica da NIC. A princípio o diagnóstico diferencial acurado entre todas estas condições parece ser fundamental para o aprimoramento do manejo individualizado e assim contribuir para a melhora da sobrevida em longo prazo dos enxertos.

De acordo com o acima citado, novos métodos diagnósticos estão sendo buscados para serem utilizados como biomarcadores de processos tóxicos, inflamatórios e fibróticos e portanto danosos aos enxertos renais. Busca-se assim ferramentas não invasivas, auxiliares ou até mesmo substitutas ao diagnóstico histopatológico. Diversos estudos demonstraram a utilidade da mensuração da expressão gênica de genes relacionados ao ataque citolítico ao enxerto em tecido renal (52), células do sangue periférico (52,54) e células do sedimento urinário (17,53,55). Mais recentemente, alguns estudos utilizando a técnica de microarranjos também foram capazes de predizer episódios de rejeição aguda

baseados em padrões de expressão gênica em relação a pacientes normais e com outras causas de disfunção (13,61,63).

No entanto é evidente a falta de estudos longitudinais robustos, essenciais para a validação de biomarcadores, que avaliem o comportamento e a modulação da expressão gênica relacionada ao desenvolvimento de episódios de rejeição e a sua resposta aos tratamentos imunossupressores. Esta lacuna nos levou a desenhar e executar o presente estudo com o objetivo de obter respostas mais claras a respeito da utilidade da metodologia molecular como ferramenta diagnóstica não-invasiva neste contexto clínico.

# Capítulo 2: Referencial Teórico

## 2.1. Transplante Renal

O transplante renal é o tratamento de escolha para uma porção significativa dos pacientes em estágio 5 da DRC sendo uma importante opção terapêutica pela melhora que propicia no estado clínico, reabilitação social e atividades laborais e econômicas (1,2). Estes pacientes têm duas opções de transplante, com doador vivo ou com doador falecido. Na primeira opção o transplante é feito entre familiares co-sanguíneos até o quarto grau ou com o cônjuge. Excepcionalmente o transplante é realizado entre indivíduos não relacionados, após autorização judicial. Este transplante com doador vivo costuma ocorrer com certa rapidez. Já na alternativa, o procedimento com o doador falecido, a alocação dos órgãos é feita principalmente pela compatibilidade no sistema HLA, não havendo assim previsão de em quanto tempo o indivíduo será transplantado. No Brasil, e em muitos outros países, o tempo médio em lista de espera é de alguns anos. As sobrevidas dos transplantes renais variam com o tipo de doador e com a compatibilidade dos antígenos leucocitários humanos (HLA), sendo em geral melhores em transplantes com doadores vivos e naqueles com melhor compatibilidade nos antígenos do sistema HLA.

Ao longo dos anos o desenvolvimento de novos fármacos imunossupressores juntamente com um aprofundamento do conhecimento dos mecanismos das doenças ou condições que levam à perda dos enxertos, vem contribuindo com as melhoras nas sobrevidas e na diminuição da incidência das rejeições agudas (3,4). Melhorias na qualidade da imunossupressão e no cuidado médico de pacientes transplantados levaram a aumentos significativos na

sobrevida de enxertos e pacientes em curto prazo. Entretanto, até o presente momento, estes avanços não resultaram em uma melhora significativa da sobrevida em longo prazo dos enxertos renais (5-7). Receptores de transplante renal requerem tratamento com drogas imunossupressoras por toda a vida ou enquanto o transplante estiver funcionante e mesmo assim permanecem com riscos de rejeições, toxicidades das drogas, recorrências de doenças que causam DRC ou infecções que ameaçam as sobrevidas dos enxertos.

### 2.2.1. A Rejeição Aguda

Tipicamente a rejeição aguda é caracterizada pela rápida diminuição da função do enxerto que é atacado pelo sistema imune do hospedeiro, o qual reconhece e ataca antígenos presentes no órgão do doador. Conforme demonstrado na Figura 1, o processo de rejeição inicia-se pelo reconhecimento dos antígenos de histocompatibilidade (aloantígenos) do doador pelos linfócitos T do receptor (8). Sumariamente o reconhecimento ocorre em um órgão linfóide secundário, onde células apresentadoras de antígeno (monócitos/macrófagos, células dendríticas e células B) estimulam e ativam linfócitos T, que entram em expansão clonal e migram para o enxerto onde irão exercer sua função efetora. No mecanismo de rejeição celular, após a estimulação dos linfócitos, pela interação receptor da célula T (TCR) com o Complexo Principal de Histocompatibilidade (CPH) juntamente com moléculas de adesão e co-estimulação, os complexos TCR/CD3 e CD4 ou CD8 tornam-se fisicamente

associados e ativam várias enzimas intracelulares denominadas tirosino-quinases. Estas elevam a concentração de cálcio intracelular e ativam várias proteínas citoplasmáticas regulatórias denominadas fatores de transcrição. Entre esses fatores destacam-se NF-kB, Oct-1 e NFAT (fator nuclear de células T ativadas), que se ligam a regiões regulatórias dos genes de várias citocinas como interleucinas IL-2 e IL-4, interferon-$\gamma$ e TNF-α. A ativação da imunidade celular seguida de resposta do tipo hipersensibilidade tardia com a ativação de monócitos/macrófagos e linfócitos citotóxicos parece ser o mecanismo final da agressão celular ao enxerto (9).

Os linfócitos T CD8+, ou citotóxicos, são responsáveis pelo reconhecimento e destruição das células-alvo. Os mediadores citolíticos melhor descritos são a perforina e a granzima, que ficam estocadas no citoplasma destes linfócitos, em grânulos semelhantes aos lisossomos e, quando as células são ativadas, migram para a membrana citoplasmática, fundem-se a ela liberando os grânulos em direção a célula-alvo. Em decorrência do ataque citolítico, a célula alvo pode morrer por necrose (caracterizada por ruptura da membrana plasmática e destruição das organelas) ou apoptose (caracterizada por condensação da cromatina, fragmentação do DNA e bolhas de membrana com citoplasma condensado). Outra via de ataque citotóxico utilizada pelas células T CD8+ é a indução de morte celular via interação Fas/Fas ligante, que leva à apoptose das células-alvo (10). Outros mecanismos ocorrem na chamada rejeição humoral, ou mediada por anticorpos. De forma resumida neste tipo de rejeição ocorre ativação de linfócitos B que se diferenciam em plasmócitos, via estimulação por

mediadores solúveis (interleucinas) produzidos por linfócitos T CD4+. Os anticorpos reconhecem seqüências específicas de aminoácidos presentes em antígenos HLA expressos na superfície das células endoteliais do enxerto, ativam a cascata do complemento e levam assim a dano da microcirculação do enxerto.

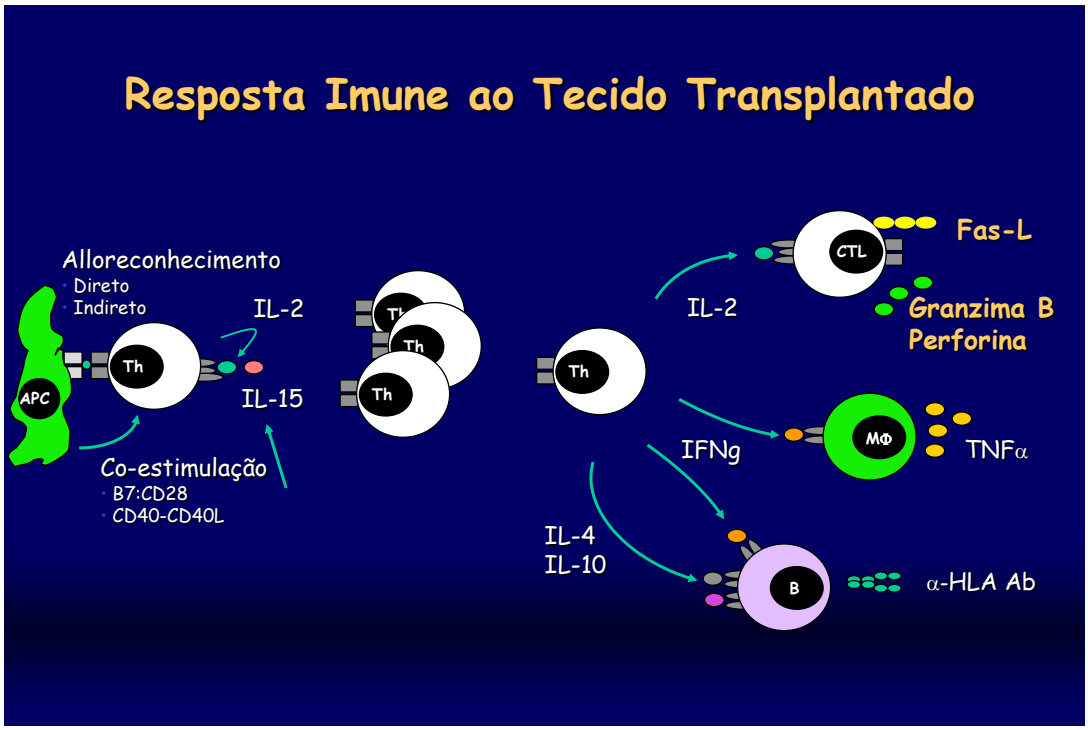



**Figura 1.** A resposta imune ao tecido transplantado. Esquema de reconhecimento aloantigênico, expansão clonal e agressão celular e humoral ao enxerto através de ativação de células B e T e suas principais moléculas efetoras.

### 2.2.2. O diagnóstico da Rejeição Aguda

Caracteristicamente o diagnóstico de rejeição aguda em enxertos renais é baseado na identificação anatomopatológica de infiltrados de células mononucleares em fragmentos renais obtidos por biópsias de enxertos com disfunção (11). O diagnóstico é feito através da padronização por um consenso internacional de nomenclaturas e critérios específicos para a caracterização histológica de rejeição do órgão, denominado classificação *Banff* (12), no qual a rejeição mediada por células T ou a rejeição mediada por anticorpos é diagnosticada com base em regras empíricas e as lesões são graduadas de maneira semi-quantitativa (13). Esta análise histológica a partir de uma biópsia renal é considerada o “padrão-ouro” no diagnóstico de rejeição do enxerto (14-16). Porém, uma vez que essa metodologia é baseada na remoção de fragmentos corticais de natureza focal intra-enxerto não pode ser considerada uma técnica muito sensível, além de estar associada às complicações como sangramento, fístula arteriovenosa e perda do enxerto, também é um método bastante agressivo e dispendioso (17-19).

O diagnóstico histopatológico da rejeição se correlaciona com a resposta ao seu tratamento e com os desfechos do enxerto. Porém sua acurácia nunca pôde ser validada devido ao fato de não haver outra metodologia independente para avaliação da existência de rejeição (20-22). Existem diversos aspectos negativos desta técnica diagnóstica. Entre eles destacam-se (a) graduação das lesões feita de maneira arbitrária; (b) variação de representatividade entre fragmentos das punções de biópsia renal; (c) concordância entre diferentes avaliadores de 10-50%

em relação a gradação das lesões e de 45-70% relacionado ao diagnóstico; (d) reprodutibilidade intra-observador de aproximadamente 80-85% (23-25).

### 2.2.3. Benefícios do diagnóstico e tratamento precoce da rejeição aguda

Os médicos com conhecimento mais aprofundado do uso clínico das drogas imunossupressoras e que sejam capazes de manejar adequadamente os atuais métodos diagnósticos são em geral capazes de reconhecer e tratar precocemente os eventos decorrentes da imunossupressão insuficiente, a rejeição aguda, ou da imunossupressão excessiva, a nefrotoxicidade, e as infecções. O exercício da prática clínica, assim como o que foi aprendido em modelos experimentais, comprovam a noção empírica de que as rejeições devem ser diagnosticadas com a maior precocidade possível e que esta precocidade tem implicação na reversibilidade do processo de rejeição (26). No entanto, são comuns situações de curso silencioso nas quais faltam métodos diagnósticos apropriados e que portanto cursam sem diagnóstico muitas vezes levando a processos irreversíveis (27). A crescente eficácia das medicações imunossupressoras tem evidenciado duas condições de importante impacto negativo nas sobrevidas tardias dos transplantes renais, quais sejam o aumento significativo nos casos de nefropatia pelo vírus polioma (28) e a nefrotoxicidade pelos inibidores da calcineurina (29). Por conseguinte, a comunidade médica envolvida com transplante está atualmente direcionada em criar estratégias que possam ser seguras em termos de propiciar baixa incidência de rejeição, de nefrotoxicidade, de infecções virais

em um balanço entre a eficácia da imunossupressão e seus efeitos colaterais nas chamadas estratégias de “minimização” da imunossupressão. Em paralelo busca-se alcançar a situação ideal, ou seja, o desenvolvimento de tolerância aos antígenos do transplante (30,31).

Assim sendo, levando-se em consideração as limitações do método “padrão-ouro” atual e a necessidade de métodos diagnósticos acurados que propiciem tratamentos precoces preconiza-se que os ensaios não-invasivos serão capazes de tornar-se ferramentas para monitorização freqüente e efetiva no período pós-transplante. Idealmente estes ensaios devem ser capazes de detectar [1] inflamação subclínica do enxerto; [2] fibrose intersticial e atrofia tubular subclínicas; [3] glomerulopatia do transplante subclínica e [4] agressão mediada por anticorpos. A base lógica para a detecção destes processos em estágio subclínico serve para a intervenção prévia ao dano causal da perda crônica de função e/ou insuficiência do enxerto (32).

## 2.3. Disfunção Inicial do Enxerto

A disfunção inicial do enxerto (DGF, do inglês *delayed graft function*), também conhecida como função retardada do enxerto, é uma das principais complicações associadas ao transplante renal no período pós-transplante imediato. Geralmente definida como necessidade de diálise na primeira semana do pós-operatório, a DGF costuma ocorrer segundo dados americanos em cerca de 20% a 50% dos pacientes receptores de órgãos provenientes de doadores

falecidos, sendo que no Brasil a sua incidência é significativamente maior (33-36). A DGF é supostamente resultado do dano isquêmico ao enxerto previamente ou durante a etapa de retirada dos órgãos, posteriormente agravado pela síndrome de reperfusão, um evento multifatorial no qual células polimorfonucleares desempenham um relevante papel (37). O papel da DGF na sobrevida do enxerto é controverso. Enquanto alguns estudos sugerem que a DGF sem rejeição não tem impacto na sobrevida em longo prazo do enxerto (38), outros apontam que a DGF e os episódios de rejeição aguda influenciam no desfecho independentemente e tem efeitos deletérios aditivos na sobrevida do enxerto (39).

No período de DGF a ausência dos parâmetros de função do enxerto, em especial da creatinina sanguínea, para guiar os procedimentos diagnósticos para a rejeição aguda torna o seu diagnóstico ainda mais complexo. Assim sendo na prática clínica a única maneira de diagnosticar-se rejeição aguda em pacientes com esta condição é pela realização de biópsias de vigilância que são realizadas usualmente a cada 7 a 10 dias até que o enxerto adquira função ou seja dado como perdido (40).

### 2.4. Rejeição Aguda Sub-Clínica

As biópsias protocolares são aquelas realizadas a intervalos pré-determinados e a despeito da função do enxerto. Com a sua implantação durante a década de 1990 inicialmente em serviços na América do Norte, foi descrita a condição que passou a ser denominada rejeição sub-clínica (RASC) na qual, por

definição, a função do enxerto é estável na presença de infiltrados linfo-monocitários característicos de rejeição aguda (41). Posteriormente foi evidenciado em um ensaio clínico randomizado que o não tratamento desta condição está associado ao desenvolvimento de rejeição crônica e nefropatia crônica do enxerto, que são as mais importantes causas de perda de enxertos em longo prazo (42-44). Em termos de freqüência, a condição rejeição sub-clínica está presente em 3-45% dos enxertos renais, na dependência basicamente da intensidade da imunossupressão utilizada (45-49).

### 2.5.1. Métodos Moleculares no Diagnóstico da Rejeição Aguda

O sistema histopatológico de uso corrente, a classificação Banff, foi formulado embasado em opiniões subjetivas de conhecedores da área, sem que tenha sido possível o benefício de uma validação independente de aspectos biológicos ou patológicos. O consenso histológico criou critérios que se correlacionam com desfechos, porém estas decisões arbitrárias deverão ser revistas diante dos novos métodos de avaliação transcripcional, presentemente denominado transcriptômica, que representa referencial independente (13). A aplicação clínica das análises moleculares é também pertinente para que o entendimento de todo o transcriptoma forneça conhecimentos mais aprofundados das bases mecanísticas da disfunção do enxerto, rejeição e tolerância. Estratégias complementares utilizando-se biomarcadores baseados em amplificação de ácidos nucléicos devem possibilitar num futuro próximo o desenvolvimento do

manuseio personalizado tanto do ponto de vista diagnóstico como de orientação terapêutica (50,51).

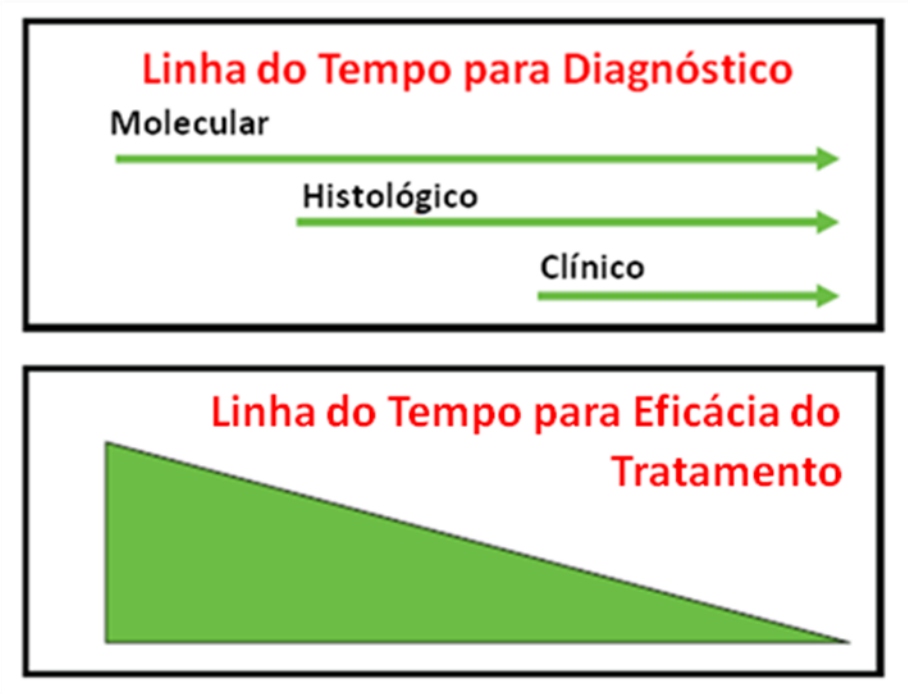
Em etapas subsequentes estas ferramentas deverão identificar condições patológicas sem a necessidade dos procedimentos invasivos e assim propiciar uma avaliação confiável do estado imune do receptor permitindo a individualização da terapia imunossupressora. O aperfeiçoamento do entendimento dos mecanismos da resposta aloimune levou a que fossem testados marcadores moleculares de genes candidatos para a avaliação do transplantado renal. Estudos recentes também focaram em padrões de expressão em testes não-invasivos de fluidos biológicos de acesso facilitado, como urina e sangue periférico (52-56).

As ferramentas diagnósticas não invasivas apresentam diversas vantagens, que incluem principalmente a possibilidade de uso frequente e sequencial facilitando a avaliação do estado imune do receptor. Assim sendo os parâmetros moleculares poderão vir a servir para direcionar a minimização da imunossupressão e sua individualização. Abordagens moleculares que incorporem um conjunto de marcadores podem vir a funcionar não apenas como uma metodologia substitutiva para o procedimento invasivo da biópsia tecidual, como também poderá fornecer informações preditivas, diagnósticas e prognósticas e prover a compreensão da fisiopatologia das diferentes causas de disfunção do enxerto (14).

Adicionalmente as avaliações moleculares propiciam a vantagem inequívoca de detectar o processo de agressão em sua fase de montagem sem

que tenha ocorrido deterioração funcional. Conforme demonstrado em diagrama da figura 2, as perturbações moleculares devem preceder não apenas a disfunção do enxerto como também as alterações histológicas (32,51).

**Figura 2.** Modelo de Linha do Tempo para Rejeição (modificado da referência 14).




Os métodos moleculares testados na arena clínica do transplante de órgãos até o presente momento são a amplificação de ácidos nucléicos pela reação em cadeia da polimerase (PCR), em especial a PCR em tempo real, os microarranjos de DNA e a detecção de microRNAs que passaremos a descrever a seguir.

### 2.5.2. PCR em tempo real

A PCR em tempo real é uma reação quantitativa altamente sensível e que permite a quantificação de transcritos raros e de pequenas variações na expressão gênica (57). O sistema da PCR em tempo real é baseado na detecção e quantificação de um repórter fluorescente. Este sinal aumenta de forma diretamente proporcional às quantidades do produto amplificado na reação. Pelo registro da quantidade de fluorescência emitida a cada ciclo, é possível monitorar a reação de PCR durante sua fase exponencial, onde o aumento significativo dos produtos amplificados se correlaciona com a quantidade inicial do produto (*template*) específico a ser avaliado (58).

O advento desta técnica quantitativa eliminou a variabilidade tradicionalmente associada a PCR convencional, semi-quantitativa, e permitiu a quantificação confiável de produtos de PCR na rotina clínica. Atualmente esta técnica é comumente utilizada para determinar a expressão gênica de mRNAs e seus níveis de expressão. As análises podem ser absolutas, como no número de cópias de mRNA pela comparação a uma curva padrão, ou relativas a um calibrador pela análise por curva padrão ou pelo método $2^{-\Delta\Delta Ct}$ (59).

Diversos mecanismos celulares que dizem respeito à sobrevivência, crescimento e diferenciação são refletidos por padrões alterados de expressão gênica e a habilidade de quantificar níveis transcripcionais de genes específicos tem sido de fundamental importância para qualquer pesquisa sobre função de um gene específico (60). A PCR em tempo real (RT-PCR) é um método de

amplificação enzimática *in vitro* de sequências definidas de cDNA, podendo ser utilizado para comparar níveis de mRNA em diferentes populações ou amostras para caracterizar padrões de expressão de mRNAs em patologias específicas. A PCR convencional, não proporciona resultados quantitativos, exceto na modalidade denominada PCR competitivo, trabalhosa, artesanal e de difícil reprodutibilidade, resolvidos com o advento dessa nova técnica (61).

O estudo transversal de Vasconcellos e colaboradores de 1998 (55) foi o primeiro a correlacionar a expressão gênica de transcritos de mRNA, pela técnica de PCR competitiva, no tecido e sangue periférico. Foram analisados os genes da Perforina, Granzima B e Fas-Ligante e foi demonstrado que a expressão gênica de moléculas de ataque citolítico em células mononucleares do sangue periférico se correlaciona com a expressão intra-enxerto destas mesmas moléculas. Assim, encontrou-se elevada acurácia diagnóstica para o evento rejeição aguda.

As análises em urina foram inicialmente realizadas por Li e colaboradores (54) estudando Perforina e Granzima B, no qual amostras de urina foram correlacionadas com biópsias renais se utilizando de uma técnica competitiva de PCR quantitativa. Níveis elevados dos transcritos destes genes na urina foram capazes de diferenciar pacientes normais de pacientes com rejeição aguda, fornecendo uma metodologia não-invasiva de diagnosticar rejeição aguda. Posteriormente, Muthukumar e colaboradores (17) também avaliando o mRNA extraído de células do sedimento urinário, pela técnica de PCR em tempo real, demonstraram elevada expressão do gene FOXP3 (Forkhead Box 3) foram

capazes de distinguir pacientes com rejeição aguda de pacientes com NCE e biópsias consideradas normais. Neste estudo a análise dos níveis de mRNA de FOXP3 foi capaz de identificar os pacientes em risco para perda de enxerto nos seis meses posteriores ao episódio de rejeição aguda.

Renesto e colaboradores (56) descreveram a utilização de uma molécula especificamente expressa em células Th1 diferenciadas, chamada TIM3 (HAVCR2 – *Hepatitis A Virus Cell receptor 2)*, como marcador de rejeição aguda em células do sedimento urinário. Níveis elevados de TIM3 foram encontrados em pacientes com RA, comparados a outras condições clínicas sugerindo o uso desta metodologia como uma ferramenta não-invasiva promissora para a avaliação das disfunções dos enxertos renais.

Esta mesma molécula foi estudada de forma pareada em tecido, células do sedimento urinário e do sangue periférico por Manfro e colaboradores (62) em pacientes com DGF e com disfunção aguda de enxertos renais. Nos casos de DGF, a avaliação dos parâmetros diagnósticos para RA apresentou acurácia de 100% nos dois compartimentos periféricos na diferenciação de necrose tubular aguda (NTA). Nos casos de disfunção aguda do enxerto as acurácias foram em torno de 90% na diferenciação entre RA de NIC, IFTA e rins normais.

Aquino-Dias e colaboradores (52) analisaram moléculas de ataque citolítico como a Perforina, a Granzima B, a Fas-ligante e uma serpina proteinase - PI-9 além do gene de células T-regulatórias - FOXP3 (Forkhead Box 3). Este estudo avaliou a utilidade desta metodologia não-invasiva em pacientes acometidos por

DGF e disfunção aguda do enxerto. Os resultados nos mostraram que a expressão aumentada destas moléculas diferenciou pacientes com NTA de pacientes com NTA e RA superimposta nos casos de DGF. Diferenciou também em casos de disfunção aguda pacientes com RA de pacientes com NTA, NIC, NCE e pacientes com biopsias protocolares normais. Como pacientes em DGF não têm atualmente um marcador não invasivo acurado de rejeição aguda, esta abordagem, se adequadamente confirmada em estudos longitudinais, seria de grande utilidade clínica.

### 2.5.3. – Microarranjos (*Microarrays*)

Estudos utilizando microarranjos para estabelecer perfis de expressão de mRNA identificaram que a rejeição aguda está associada com importantes perturbações na expressão de múltiplos genes, incluindo aqueles envolvidos no ciclo celular, metabolismo e imunidade (14). No estudo pioneiro de Sarwal e colaboradores (63) demonstrou-se que testes moleculares poderiam evidenciar um painel de genes relacionados a resposta imunológica contra o enxerto. Neste estudo, biópsias de pacientes com rejeição aguda que eram indistinguíveis por análise histológica convencional, revelaram diferenças na expressão gênica associada com diferenças de componente imunológico e celular e curso clínico. Foram encontrados também aglomerados de células B em biópsias associadas com rejeição severa de enxerto, o que sugere um papel essencial das células B na rejeição aguda.

Flechner e colaboradores (64) desenvolveram o primeiro estudo utilizando esta metodologia em células mononucleares do sangue periférico concomitante à amostras de biópsia. Seus resultados foram muito animadores, uma vez que a assinatura transcripcional foi capaz de diferenciar RA, disfunção aguda sem rejeição e transplantes com função estável sem histórico de rejeição. Uma peculiaridade deste estudo foi a demonstração de que mesmo que esta assinatura molecular em sangue periférico tenha sido capaz de distinguir entre RA e pacientes com função estável, os padrões de expressão gênica foram muito diferentes dos padrões expressos no tecido.

Em 2007, estudo de Brouard e colaboradores (65) avaliou a utilização da monitorização molecular como ferramenta não-invasiva de tolerância operacional em pacientes transplantados renais. Este estudo teve como objetivo a identificação de biomarcadores de tolerância operacional em sangue periférico com intuito de utilizá-los para a determinação da frequência deste estado em pacientes com função renal estável na presença de imunossupressores. Em um primeiro momento, foram segregados 49 genes relacionados à tolerância e posteriormente analisados por microarranjos e PCR em tempo real e 33 deles conseguiram diferenciar os fenótipos de tolerância e rejeição crônica com alta especificidade. A assinatura genética demonstrada sugere um padrão de redução de sinalização co-estimulatória, quiescência imunológica, apoptose e respostas de células T de memória, identificando no sangue periférico de transplantados renais um grupo de genes associados à tolerância operacional que pode vir a ser útil

como ferramenta não-invasiva para guiar a administração de medicação imunossupressora.

Em uma publicação mais recente, Reeve e colaboradores compararam a abordagem molecular com a histopatológica (13). Este estudo sugere que a abordagem molecular é mais fidedigna em representar o estado de agressão imunológica ao enxerto do que a abordagem histológica, uma vez que as bases diagnósticas são empíricas e observacionais (tratamento-dependente), sem um *background* explicativo da biologia mecanicista responsável pelo dano acarretado ao enxerto. Portanto, este estudo sugere que as discordâncias entre esta metodologia e o diagnóstico através da técnica padrão-ouro são necessárias, uma vez que, segundo o autor, esta nova metodologia surge como uma técnica mais poderosa de diagnóstico do estado imune do paciente. A significância destes resultados no contexto de monitorização de pacientes submetidos a transplante renal podem explicar a falha de mais de uma década de trabalhos que analisaram em células do sangue periférico a ativação de antígenos baseada nos achados em biópsias de enxertos com rejeição e outros modelos imunes. É possível que o perfil de expressão gênica dos linfócitos do sangue periférico represente a adequação da imunossupressão de tal forma que em pacientes que venham a rejeitar reflitam a imunossupressão insuficiente comparados com pacientes transplantados com função renal estável, sem mecanismos operantes de agressão aloimune.

Na metodologia dos microarranjos o fato de se demonstrar que os transcritos ocorrem de maneira coordenada sugere que um limitado número de transcritos de genes seja necessário para a classificação de rejeição versus não-rejeição. Por fim um fato muito importante a ser considerado ao se desenhar uma ferramenta não-invasiva para monitorização do enxerto é o custo da metodologia. Presentemente os microarranjos são muito onerosos para utilização na rotina clínica, onde a análise de alguns genes de interesse por PCR em tempo real parece ser uma abordagem mais viável (32).

### 2.5.4. MicroRNA's

Um importante avanço na biologia das células nos últimos anos foi a descoberta dos microRNAs (miRNAs) (66). miRNAs são RNAs pequenos (~ 19-25 nucleotídeos), conservados na evolução das espécies, de ocorrência natural, abundantes, não codificadores e que regulam a expressão gênica, primariamente por repressão translacional ou por degradação de RNA mensageiro (mRNA) (67). O primeiro miRNA foi descrito em 1993 (68,69), porém atualmente centenas já foram clonados e previstos pela bioinformática (70). Estudos recentes comprovaram que um único miRNA é diretamente responsável pela repressão de centenas de proteínas e regulando os níveis de milhares de outras (71). Diversos processos biológicos como desenvolvimento (72), proliferação celular, diferenciação, apoptose, metabolismo e oncogênese (73) são descritos como sendo regulados por miRNAs. Dados recentemente publicados sugerem que os

miRNAs desenvolvem um papel crítico na regulação do desenvolvimento de células imunes e na modulação das respostas imunes inatas e adaptativas (74-77).

No transplante renal uma questão crítica e não resolvida é a base mecanicista para as principais perturbações na expressão gênica durante um episódio de RA. Recente estudo publicado por Anglicheau e colaboradores (78) investigou a associação da rejeição aguda com alterações significativas na expressão de miRNAs em tecido de enxertos renais e se estes padrões de expressão intraenxerto de miRNAs são capazes de diagnosticar RA e predizer função renal. Como resultado, encontrou-se que os miRNAs que estão hiperexpressos em biópsias com RA (miR-142-5p, miR-155 e miR-223) estão também presentes em altos níveis em células mononucleares do sangue periférico em comparação com os miRNAs hipoexpressos (miR-30a-3p, miR-10b ou let-7c) em biópsias sem RA. Outro achado importante deste estudo, foi que dos 53 miRNAs diferencialmente expressos entre biópsias de RA e normais, 43 encontravam-se hipoexpressos e somente 10 estavam hiperexpressos. Nas conclusões deste estudo, os padrões de expressão intraenxerto são preditivos do estado do aloenxerto, e juntamente com os dados existentes de que miRNAs são estáveis (79), abundantes e podem ser examinados em tecidos fixados em formalina (80), segue-se a idéia de que a expressão de padrões de miRNA possa vir a ser de valor como biomarcadores clínicos do transplante renal onde claramente mais estudos são necessários para estabelecer-se o valor e a potencial aplicabilidade desta técnica.

## 2.6. Conclusões

Existe a expectativa de que os microarranjos venham a desempenhar papel crucial no transplante de órgãos em assuntos relacionados à identificação de mecanismos moleculares de rejeição aguda, injúria crônica, efeito de toxicidade das drogas imunossupressoras e tolerância. É também esperado que eles ajudem a identificar novos alvos de drogas para um tratamento com imunossupressores pós-transplante mais personalizado. Mais importante, se espera que os microarranjos ajudem a identificar biomarcadores, para uso não-invasivo, para o diagnóstico clínico de rejeição e de tolerância. Em uma abordagem clínica, existem duas razões primárias para a utilização de um número pequeno de genes para o monitoramento clínico por PCR em Tempo Real. A primeira, PCR em Tempo Real é consideravelmente menos onerosa para se testar alguns genes, e pode ser realizada rapidamente e na maior parte dos laboratórios. Microarranjos consistem em arranjos de genes customizados e são úteis para testar-se múltiplos genes, em geral centenas. Segunda, resultados obtidos da análise por microarranjos são complexos e requerem muito tempo e pessoal altamente treinado para sua interpretação ao passo que os dados provenientes de PCR em tempo real podem ser economicamente replicados e sua análise é estatisticamente mais simples e rápida. Adicionalmente, a PCR em tempo real é mais sensível para pequenas mudanças na expressão gênica. A comparação das tecnologias de microarranjos e PCR em tempo real identificando seus pontos fortes e fracos foi proposta por Kahtri e colaboradores (81) e está apresentada na tabela 1.

**Tabela 1. Comparação das tecnologias de Microarranjos e PCR em Tempo Real**

| Microarranjos | PCR em Tempo Real |
|---|---|
| Custo efetiva quando rastreando dezenas de milhares de genes, relativamente dispendiosa para rastreamento de apenas dezenas de genes | Custo efetiva testanto algumas dezenas de genes no máximo; cara e consome tempo para testar algumas centenas de genes |
| Tecnologia relativamente nova na maioria das clínicas e requer pessoal altamente treinado para análise dos dados | Análise de dados simplificada e de fácil aplicabilidade na rotina clínica |
| Sofre de baixa sensibilidade devido a impossibilidade de detecção de transcritos pouco abundantes em uma amostra | Alta sensibilidade permite a detecção de transcritos pouco abundantes em uma amostra |
| Sofre de baixa especificidade devido a hibridização cruzada assim como erros no desenho das sondas, o que também limita a detecção de transcritos splice-alternativos | Alta especificidade; possibilidade de desenhar primers para identificação de transcritos splice-variantes |

Modificado de 81.

O desenvolvimento de técnicas moleculares capazes de fornecer um painel da atividade imunológica responsável pela agressão ao enxerto de forma não-invasiva sugere em futuro próximo a possibilidade de otimização e individualização do tratamento com drogas imunossupressoras. Situações clínicas como a DGF, a RASC, o desenvolvimento de fibrose (IFTA) e outras, são situações onde a monitorização dos processos nocivos ao enxerto é feita através de biópsias, são eventos nos quais a monitorização molecular não invasiva poderá apresentar enorme valor diagnóstico.

Enquanto o potencial impacto clínico das assinaturas de expressão gênica que podem predizer a RA e monitorar a imunossupressão é claro, as possíveis contribuições para o entendimento da biologia do transplante também são importantes considerações. Por conseguinte, o objetivo máximo da assinatura de expressão gênica é identificar genes específicos e associá-los a rotas de mecanismos celulares mediadores de rejeição, lesão e reparo tecidual, imunossupressão e tolerância. Com o conhecimento dos mecanismos de ação é possível que se desenvolvam novas drogas e abordagens mais eficazes que

permitam a monitorização segura do curso clínico e de eventuais estados de tolerância ao enxerto.

Por fim, considera-se que as análises moleculares surgiram para revolucionar o entendimento funcional da “aloimunogenômica” no transplante de órgãos, descobrindo genes que possam servir como marcadores de estados e de progressão de doenças, além de estratificar risco para injúria orgânica e sistêmica.

# Capítulo 3: Justificativa

Com o advento de fármacos imunossupressores mais potentes e específicos (baseados nos mecanismos de atuação no sistema imune), a sobrevida em curto prazo dos transplantes renais já há muitos anos demonstra melhora significativa. Episódios de rejeição aguda são progressivamente menos freqüentes, entretanto, não ocorreu melhoria significativa na sobrevida dos enxertos renais, em longo prazo, nas últimas três décadas. A cicatrização dos enxertos, que acaba por deteriorar a função renal parece ser fruto de três processos básicos: [1] o processo imunológico de agressão ao rim transplantado seja como decorrência da RA ou por mecanismos aloimunes subclínicos; [2] a toxicidade por utilização de inibidores de calcineurina e [3] a inflamação determinada pelo vírus polioma. As ferramentas diagnósticas de que se dispõe atualmente não satisfazem a necessidade de acesso ao estado imune do receptor de maneira não invasiva e fidedigna. A técnica padrão-ouro atual, a biópsia renal, é pouco sensível e talvez seja tardia demais para orientar o manuseio da imunossupressão. Desde o fim da década de 1990, a metodologia de diagnóstico molecular vem sendo proposta como uma ferramenta não-invasiva para a avaliação das complicações imunológicas pós-transplante.

Com este estudo objetivamos auxiliar na consolidação da utilização destas técnicas como ferramentas auxiliares no acompanhamento de pacientes transplantados renais, visando um diagnóstico mais acurado e precoce dos episódios de rejeição aguda.

# Capítulo 4: Objetivos

## 4.1. Objetivo Geral

Consolidar a mensuração do mRNA pela técnica de PCR em tempo real como instrumento para o diagnóstico não-invasivo da rejeição aguda de transplantes renais.

## 4.2. Objetivos Específicos

1. Estudar longitudinalmente a cinética da expressão gênica pós-transplante renal imediato;
2. Avaliar as modificações da quantificação gênica por RT-PCR em resposta aos tratamentos para a rejeição aguda (RA) de transplantes renais;
3. Identificar entre os genes candidatos os que apresentam melhores parâmetros para a monitorização da imunossupressão em pacientes transplantados renais.

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# Capítulo 6: ARTIGO “A longitudinal study of the mRNA transcriptional evaluation in human kidney allograft dysfunction.”

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**A longitudinal study of the mRNA transcriptional evaluation in human kidney allograft dysfunction.**

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**Abstract**

The aim of the present study was to longitudinally evaluate the diagnostic accuracy of the transcriptional analyzes of kidney allografts. Thirty-six kidney transplant recipients were evaluated up to three months after grafting. Peripheral blood samples for the real-time polymerase chain reaction analyzes of the Perforin and TIM3 genes were drawn at days 3, 4-6, 9-11, 14-16, 19-21, 24-26, 29-31, 44-46, 59-61 and 89-91 post-transplantation. Indication and surveillance biopsies were obtained to evaluate acute graft dysfunction or during DGF, respectively and interpreted using the Banff 2007 classification. Thirteen patients presented 16 episodes of acute rejection. Recipients with rejection had significantly higher levels of TIM3 mRNA transcripts as compared to those without rejection (median gene expression 153.7 and 40.1 respectively, $p < 0.001$). Also, Perforin gene expression was higher in patients with rejection (median gene expression 136.6 and 46.5, $p < 0.001$). Receiver operating characteristic (ROC) curves showed that the area under the curve (AUC) for the TIM3 gene was 0.752 (95% CI: 0.653 - 0.852). Perforin gene mRNA expression provided an AUC of 0.733 (95% CI: 0.580 to 0.809). Overall accuracy of gene expression was 77% for the TIM3 gene and 66% for the Perforin gene. Combined accuracy reached 82%. Negative predictive values (NPV) were 96% for all three analyzes. Gene expression was significantly modulated by rejection treatment decreasing 60% (TIM 3) and 48% (Perforin) compared to the pre-rejection samples. In conclusion the longitudinal approach showed that gene profiling might be very useful in ruling out acute rejection and ascertain the efficacy of its treatment.

**Key-words:** Kidney transplantation, acute rejection, gene expression, diagnosis, mRNA

## Introduction

Kidney transplantation has become the therapy of choice for most patients with end-stage kidney disease. In the last decades significant improvements occurred in the first year post transplantation outcomes but, despite this early success, long-term survival of patients and allografts has not improved significantly (1-2).

Acute rejection (AR), defined as graft aggression resulting from the recipient's immune response to the donor antigens expressed in grafted organs, is a major immunological event and may influence short and long term outcomes. It typically occurs during the first months following renal transplantation and its diagnosis is suspected by an increment in serum creatinine and confirmed by histological analysis of graft tissue (3-6). A major difficulty in current practice is that biomarkers presently used in renal transplantation are clearly not accurate to differentiate AR from other causes of graft dysfunction, such as acute tubular necrosis (ATN), calcineurin inhibitors nephrotoxicity (CIN) and viral or bacterial infections (7). No single satisfactory method of diagnosing AR is currently available. Instead, combined methods are used for post-transplantation monitoring. Increased creatinine levels signal kidney dysfunction and are used as an indication for biopsy. Unfortunately, rising serum creatinine levels are only evident after graft damage has occurred and its sensitivity in detecting dysfunction has been discussed extensively (8-9). Other methods such as duplex doppler ultrasonography detect rejection with widely varying sensitivity of 9-76% and is used as an adjunct to the measurement of serum creatinine and immunosuppressive drug levels (10). Moreover, this approach recognizes rejection at a relatively advanced stage of the immune process and tissue injury, and fails to diagnose subclinical acute rejection (11).

The histological examination of allograft tissue remains the gold standard for the etiologic diagnosis of allograft dysfunction. Recent refinements have reduced but not eliminated biopsy-associated complications, such as hematuria, anuria, perirenal hematoma, bleeding and shock, arteriovenous fistulas, and graft loss (11-13). Besides, sampling errors, poor reproducibility in interpretation of the histological diagnosis and the focal characteristic of the inflammatory process of rejection pose additional problems leading to the need of multiple samples to increase diagnostic accuracy without however assuring high accuracy. Also to be considered are the elevated costs of the biopsy procedure (14-17). By the other side protocol biopsies displaying features of inflammation and occurring in grafts without evidence of disease, the so called subclinical rejection (SCR), have been associated with chronic pathology and eventual renal transplant loss (18).

The analysis of molecular changes during the early post-transplantation period provides insights into the activity of the immune system in response to transplantation (19). Noninvasive tools have several advantages including frequent and sequential assessments of recipient's immune status. The recent developments in the field of molecular monitoring of the solid organ transplant recipients have focused on noninvasive tests of easily accessible biological fluids, such as urine and peripheral blood (1,7,19-24). Results obtained with hypothesis-driven candidate messenger RNAs (mRNA) expression patterns ascertained in urine and blood have been quite impressive in cross sectional studies showing that molecular perturbations may precede not only graft dysfunction but also histological changes. Importantly, molecular parameters may become useful to guide individualization strategies of the immunosuppressive therapy (11). However it must be recognized that most of the studies employing these tools are cross-sectional and their

validation as biomarkers still require the demonstration of adequate accuracy in longitudinal follow-up studies (25).

In the present study we undertook a longitudinal analyzes of TIM3 and Perforin mRNA gene expression in order to evaluate their utility as non-invasive biomarkers of allograft responses in kidney transplantation testing the hypothesis that quantitative transcriptional analyses could serve as accurate biomarkers of acute rejection following kidney transplantation.

## Materials and Methods

### *Patients*

Thirty-six kidney transplant recipients were enrolled in a longitudinal observational study. They were all informed of the study proposal and agreed in participate by signing an informed and written consent. Blood samples were sequentially draw at days 3, 4-6, 9-11, 14-16, 19-21, 24-26, 29-31, 44-46, 59-61, 89-91 post transplantation. Acute rejection was diagnosed by histopathological analysis of graft biopsy performed in the clinical situation of graft dysfunction, determined by a confirmed rise in serum creatinine, or as a surveillance biopsy in patients with delayed graft function (DGF). DGF was defined by the need of dialysis within the first week after transplantation. Based on the occurrence of acute rejection patients were classified as either rejectors or non-rejectors and their gene expression was studied. The study was approved by the research and ethics committee of Hospital de Clínicas de Porto Alegre, accredited by the National Research Council of the Brazilian Ministry of Health, and registered at the Office for Human Research Protection – OHRP-USDHHS.

***Immunosuppression and anti-rejection therapy***

Immunosuppression was initiated pre-operatively in all patients who received a 500 mg dose of methylprednisolone transoperatively and were maintained with a combination of prednisone, sodium mycophenolate and tacrolimus or cyclosporine. Patients considered as high-risk for rejection received induction therapy with Thymoglobulin® and patients with post-operative oliguria or anuria received therapy with Basiliximab® within 24 hours of the transplant surgery. Rejections were treated with a 3 day course of methylprednisolone 500 mg IV. Steroid resistant rejections and those with initial Banff classification Banff IIA or higher were treated with a 10-14 day course of Thymoglobulin® or OKT3®.

***Sample Handling and Design of Primers and Probes***

Peripheral blood samples were drawn in EDTA-containing tubes. Peripheral blood leukocytes (PBL) were obtained through erythrocyte lysis with a hypotonic buffer and stored at -80ºC. RNA isolation was performed using the QIaAmp RNA Blood Mini Kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturer´s instructions. Total RNA quantification were made using the Nano'drop® 1000 Spectrophotometer v.3.7 (Thermo Fischer Scientific, Wilmington, DE, USA) and RNA purity was observed as a ratio of absorbances at two different wave lengths (260/280 nM). Only samples with optical density ratio higher than 1.7 were analyzed. Total RNA was reverse transcribed to cDNA using the cDNA High Capacity Kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer´s instructions, to a final volume of 20uL and stored at -20$^{o}$C.

The 5’ nuclease assay was performed using the ABI 7000 Sequence Detection System and TaqMan Universal PCR Master Mix, composed by AmpliTaq Gold® DNA polymerase, Amperase UNG, passive reference (ROX), buffer and dNTP´s (Applied Biosystems, Foster City, CA, USA). The design and synthesis of the primers and fluorogenic probes for Perforin (ID: Hs 00169473_m1; GenBank reference: 5551) and TIM3 (ID: Hs 00262170_m1; GenBank reference: 84868) mRNA were made by TaqMan® Gene Expression Assays (Applied Biosystems, USA) and had already been tested and validated previously by the manufacturer. 18s rRNA, due to its low inter sample variability, was used as an endogenous control (Taqman PDAR Endogenous Control, Applied Biosystems). Gene Expression Assays consisted of 20 times concentrated (360uM) mix of PCR primers and Taqman® MGB (Minor Groove Binding) probes. These assays are designed for the detection and amplification of specific genetic sequences. All primers utilized are intron-spanning to avoid genomic DNA amplification (Gene Expression Assays/Custom Primers and Probes; Applied Biosystems, USA). The Taqman® probes were labeled with FAM (6-carboxyfluorescein) as the reporter at the 5’ region and with TAMRA (6-carboxytetramethyl rhodamine) as the quencher at the 3’ region dyes, except the endogenous control 18s rRNA that was labeled with the dye VIC as the reporter. Gene expression relative quantitation was measured as a raise in fluorescence, resulting from amplification and probe degradation. The cycle in which the fluorescence exceeds the detection threshold is called Threshold Cycle (Ct). More specific template in a sample results in an earlier exceeding fluorescence. The third day post transplant sample was used as calibrator. For the diagnosis of rejection and analysis of anti-rejection therapy the last sample before the beginning of rejection treatment was used. The analyses of amplified

products were performed by the relative quantification method $2^{-\Delta\Delta Ct}$, which describes alterations to the target gene expression relative to a reference sample (26).

### *Statistical Analyses*

Descriptive analyses, means ± standard deviations and distributions are shown. Receiver operating characteristic (ROC) curves and non-parametric Mann-Whitney and U-Wilcoxon tests were used for statistical analysis. Fisher's exact test was used to compare qualitative variables and Non-parametric Wilcoxon signed-rank test was used to compare gene expression levels pre and post treatment. A P level lower than .05 was considered statistically significant.

## Results

Thirty six kidney recipients transplanted between January 2008 and March 2009 were prospectively evaluated during the first 90 post-transplant days. Acute rejection episodes either presented by graft dysfunction or during the period of DGF were confirmed by graft biopsies and patients were divided into two groups, the group of those who had at least one rejection episode ("rejectors") and those who did not have detected rejections episodes ("non-rejectors"). The main demographic data of the groups are shown in Table 1. No differences with statistical significance were found in the comparison of gender, race and immunosuppressive regimen distributions. Mean age, panel reactive antibodies, cold ischemia time, HLA matching (loci A, B, and DR) and serum creatinine 30 days after transplantation did not present significant differences. Rejectors presented a higher percentage of deceased donor recipients, 56.5% versus 23.0% in non-rejectors, respectively

(P < 0.05). DGF was more frequent in the group of rejectors as compared to the non-rejectors, the incidences were 76.9 % versus 26.0%, respectively (P < 0.005).

***Gene expression analyses***

Twenty three samples from 13 recipients who had 16 acute rejection episodes were compared to 237 samples that include all the samples from 23 patients without rejection (n=169) and the post-treatment samples of the patients with rejection (n= 68).

Recipients with rejection had significantly higher levels of TIM3 mRNA transcripts as compared to those without rejection. The median gene expression values were 153.7 and 40.1 respectively (Mann-Whitney, P < 0.001). A statistically significant difference was also observed in the median mRNA expression of the Perforin gene, 136.6 and 46.5 were the values for rejectors and non-rejectors respectively (Mann-Whitney; P < 0.001).

Receiver operating characteristic (ROC) curves were generated to analyze the diagnostic parameters of mRNA gene expression (Figure 1). The area under the curve (AUC) observed for the TIM3 gene was 0.752 with a 95% confidence interval ranging from 0.653 to 0.852. Increment of 30% in gene expression, relative to the postoperative calibrator, was chosen as a threshold for the determination of the diagnostic parameters that were sensitivity: 70%; specificity 78%; positive predictive value (PPV) 23%, negative predictive value (NPV) 96%, and accuracy 77% (Fisher's exact test; P < 0,001). Perforin gene mRNA expression provided an AUC of 0.733 with a 95% confidence interval from 0,658 to 0,809. The diagnostic parameters for the Perforin gene were: sensitivity 78%; specificity 66%; PPV 18%; NPV 96% and accuracy 66% (Fisher's exact test; P < 0.001) (Table 2).

Among the 16 episodes of acute rejection observed in this study, we found an increased expression in one or both genes in each event. Regarding the 23 samples obtained in these 16 events, 20 had a raise in their expression in one or both genes (86%). Among these 20 samples, 13 had an increased expression in both genes (65%), while 3 had isolated increased expression of TIM3 (15%) and 4 samples had isolated increased expression of Perforin (20%). Combined gene analyzes (TIM3 and Perforin) using the same cut-offs as for single gene analyses are also shown in table 2. Scores were applied to the samples so they were assigned as double positive meaning high expression of both genes. The combined analyzes resulted in a sensitivity of 60%, specificity of 84%, PPV of 27%, NPV of 96% and an accuracy of 82% for the double positive indicating the presence of acute rejection (Fisher's exact test; $P < 0.001$). TIM3 parameters were sensitivity of 70%, specificity of 78%, PPV of 23%, NPV of 96% and an accuracy of 77% while Perforin parameters were sensitivity of 78%, specificity of 66%, PPV of 18%, NPV of 96% and an accuracy of 66% (Fisher's exact test for both genes; $P < 0.001$).

***Effects of anti-rejection therapy***

In the present study acute rejection was diagnosed in a mean of 9 days post-transplantation (variation: 6 to 14 days). The mean time for the molecular diagnosis was 5 days (variation: 4 to 7 days) significantly shorter than the time required for the clinical diagnosis (Paired T test, $P < 0.001$). Gene mRNA comparison pre and post rejection treatment was also analyzed. In the 13 patients with acute rejection the last sample taken prior to treatment was used as calibrator for the determination of the post-treatment gene expression. The median of the gene expression of TIM3 pre-treatment was 189%, related to

the calibrator, and the post-treatment median was 29% (Wilcoxon rank test, $P < 0.01$). The median of the gene expression for Perforin pre-treatment was 166% and the post-treatment median was 18%, related to the calibrator (Wilcoxon rank test, $P < 0.01$) (Figure 2).

**Discussion**

In the last decade molecular techniques, specially the real-time polymerase chain reaction technique, have been evaluated for the non-invasive diagnosis of renal allograft dysfunction, with emphasis in the detection of acute rejection and analyzes performed in either peripheral blood or urine have produced accurate diagnostic parameters for such purpose (19-24). Among the advocated advantages of the transcriptional approach are to diagnose rejection by noninvasive means and obviate the need for the allograft biopsy; anticipate the subsequent development of rejection before the development of tissue injury; prognosticate the outcome of an episode of rejection, and responsiveness to anti-rejection therapy; prediction of subsequent allograft function; help in developing mechanism-based therapy and facilitation of individualization or optimization of immunosuppressive drug therapy including weaning or reintroducing the therapy (11). However, up to this moment, the great majority of the reported studies are cross-sectional in design and do not provide a complete evaluation such as can be obtained with a longitudinal approach. Another weakness of the available studies are the type of controls used which might not be the most appropriate in the sense that they are composed by patients with clear cut different conditions that lead to graft dysfunction and not by patients who rejected outside of the rejection episode. Moreover one can argue that the current gold-standard, the

histopathology analyzes by the Banff classification (14), used has many faults and must be interpreted with caution (27).

A number of different genes have been evaluated either in the peripheral blood (19,28-29) or in the urine (1,7,30) and they are generally well correlated in both compartments and with tissue (22). In the present work we report the longitudinal expression of well validated molecules namely Perforin and TIM 3, both expressed in cytotoxic T lymphocytes (CTLs) that were shown to play an important role in tissue destruction during rejection (1,31-32). Perforin is a molecule stored and subsequently secreted by effector CTL granules leading to pore formation in the target-cell membrane and ultimately causing cell death (33). TIM-3 is a type I membrane protein preferentially expressed on terminally differentiated Th1 cells which seem to be central in the mechanisms of allograft rejection (34-37). Specifically, TIM-3 has been associated with autoimmune diseases, tolerance induction and to the regulation of Th1 immune responses (38-40). Recent studies had shown that non invasive (peripheral blood and urinary sediment cells) TIM3 and Perforin gene expression are upregulated during acute rejection episodes of kidney grafts. In such studies it was demonstrated that patients with acute rejection express excessive mRNA amounts of TIM3 and Perforin as compared to patients with other causes of graft dysfunction and without acute rejection (1,20-24). The known functional attributes of TIM 3 and Perforin provided the rationale for the evaluation of their mRNA levels in peripheral blood leukocytes as markers of acute rejection.

We also analyzed the diagnostic usefulness of TIM3 and Perforin mRNA gene expression measurements in the peripheral blood for the diagnosis and prediction of acute rejection. In contrast to previous studies measuring gene expression in samples collected at

single time-points, mRNA gene expression was analyzed sequentially during the first 3 months post transplantation. As shown in previous studies we found a significantly higher gene expression, for both genes, during the rejection episodes. Moreover and perhaps more importantly it was found that patients who subsequently presented acute rejection were identified by the molecular evaluation in a mean of 4 days before the clinical diagnosis.

Simon et al. in the only longitudinal study published up to now demonstrated that rejection could be identified a median of 11 days before clinical diagnosis (20). In our study rejectors were identified 4 days before the clinical diagnoses. The difference between ours and Simon's study is that in ours rejection episodes occurred earlier, in a mean of 9 days post-transplantation, as compared to Simon's in which rejection episodes occurred latter. It is however important to emphasize that in both studies the molecular approach allowed an earlier diagnosis.

Based on increased mRNA gene expression it was found that the diagnostic accuracy in these sequential evaluations were lower than those reported in the prevalence studies. Accuracy for individual gene analyzes were 66 and 77% for the Perforin and TIM3 genes and was substantially increased to 82% by the combined analyzes. Importantly, and in accordance to Reeve et al. (27), the negative predictive values were elevated indicating that rejection episodes would hardly occur in the absence of increased gene expression. Contrary to our hypothesis the positive predictive values lower than expected suggesting that, the PPV found in cross-sectional studies might be misleading, at least for the genes here studied (19,20,22-23). Actually, Simon et al also reported PPV that although higher than ours were significantly lower than those reported in the cross-sectional studies (20). Also in the study by Simon and collaborators, the PPV were increased by their approach in

data analyses. Time separations, per period after transplantation and using different cutoff values for each time interval, lead to optimized results but it certainly makes more difficult the clinical applicability of the results (20).

There are some reasons why the results here presented differ in their PPV from the previous reported studies, including ours (22-23). Prevalence studies will peak up a well defined situation (eg. acute rejection) which is compared to other equally well defined clinical situations, all retrospectively diagnosed based upon the histological criteria. In this approach variations of gene expression are not detected. Contrariwise when patient's samples are collected sequentially variations of expression will be detected and when these variations are not related to alloimmune activation or are not relevant in terms of graft aggression they will necessarily decrease the positive predictive value of the test. Viral infections, which may be clinically silent, can elicit a TH1 response that involves many of the genes that participate in the acute rejection phenomena (41) and subclinical rejection, which will not be detected unless protocol biopsies are performed (42-44) are among the reasons why gene expression variation may occur overtime after transplantation. In fact previous studies showed that gene transcription in surveillance biopsy infiltrate was phenotypically similar to that of infiltrate from clinical rejection biopsies with both perforin and granzyme B expression up-regulated, but at a slightly lower level in subclinical as compared to clinical biopsy infiltrate (44). Moreover lending support to the relevance of these subclinical processes Rush et al accomplished a randomized prospective study to demonstrate that improved graft function and survival could be achieved in patients with surveillance biopsies and treatment of subclinical rejection (18). Also, as shown by Manon et al, polyoma virus infection has been shown to display a molecular signature that up to

now is indistinguishable from the one obtained in graft rejection (41). Another possible reason for the false-positive results is the power of the molecular tool. It is quite possible that the RT-PCR peaks up very small and possibly not relevant increments in gene expression that do not produce graft aggression. Alternatively these very low levels of expression may in fact be detrimental to the graft leading to progressive fibrosis (27).

Moreover it can be argued that the low PPV rely on the recognized inadequacy of the gold-standard that is currently used and widely accepted in clinical kidney transplantation. The histological analyzes of the renal tissue has many drawbacks, including the fact that histological diagnose is arbitrary and opinion based and correlates with treatment response and graft outcome, but its accuracy has never been validated because no independent system for assessing rejection exists. Additionally, histology has two main biases: subjectivity assigning lesion grades and variation between biopsy cores. These problems limit reproducibility and an agreement between two pathologists on lesions scoring is 10-50% and on diagnosis 45-70%, and even intra-observer reproducibility is only approximately 80-85% (27,45-50). As in the present study protocol biopsies were not undertaken one could argue that subclinical rejections or viral infections might account by some of the false positive results. However it must be considered that the utility of a test does not rely only in its PPV but the NPV is also important. In this regard we can infer that the molecular approach to graft immune injury can be very useful as it permits an accurate diagnosis of the absence of rejection since a negative quantification gives a post test probability of excluding rejection that is higher than 95%.

Another important finding of the present study is the down regulation of gene mRNA expression observed in response to rejection treatment. Both Perforin and TIM 3

mRNA transcripts amounts decreased significantly upon rejection therapy. Similar results have already been presented by Strehlau and collaborators in renal tissue (51) and by Simon et al. and Vasconcellos et al. in peripheral blood leukocytes (19,20). Taken together these findings suggest that the non-invasive gene expression assays might became useful in the monitoring of acute rejection immunosuppressive treatment efficacy. Whereas molecular features are suppressed by treatment more quickly than are histopathology lesions, suggesting that they probably reflect the true state of the tissue more reliably than the histopathologic lesions, which can linger despite successful treatment (27).

The accurate non-invasive assessment of the renal allograft status, mainly the alloreactivity, is determinant in the successful management of the renal allograft. Acute graft dysfunction is known to have a strong detrimental effect on graft survival (4). In this sense the demonstration of elevated levels of cytotoxic genes transcripts must be seen as a potentially harmful in the critically important early post-transplantation period. The question remains as to whether this warrants the use of increased immunosuppression or even pre-emptive anti-rejection therapy. The main advantage of the molecular approaches, as compared with conventional post-transplantation monitoring techniques, is the potential for earlier intervention by the physician, prior to substantial organ damage. Since acute rejection is a major risk factor for later chronic rejection, earlier intervention may result in prolonged graft function and reduction in other costs such as hospitalization, expensive rejection treatments, dialysis and re-transplantation. Considering the lack of uniformity in transplant center procedures, the actual implementation of this system in the clinic depends on a series of unmet validation steps such as independent center judgments on the value associated with earlier intervention. This decision is likely to be made more easily in the

future through expansion of research into this area such as further testing in randomized studies (20,25).

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**Table 1.** Demographic and transplant data

| Parameter | **Rejectors (n=13)** | **Non-rejectors (n=23)** | **P value** |
|---|---|---|---|
| Recipient gender (male/female)[a] | 10/3 | 16/7 | .367 |
| Age (years)[b,c] | 45 ± 11 | 39 ± 13 | .711 |
| Race (caucasian/non-caucasian) | 11/2 | 21/2 | .459 |
| Panel reactivity antibodies (%)[b,c] | 1.8 ± 6 | 3 ± 10 | .475 |
| Cold ischemia time (hours)[b,c] | 19.7 ± 5 | 17 ± 6 | .845 |
| Donor (living/deceased)[a] | 03/10 | 13/10 | < .05 |
| HLA mismatches (A, B, DR)[b,c] | 3.5 ± 1 | 2.8 ± 1 | .197 |
| DGF cases and (percentage)[a] | 10 (76.9) | 6 (26.0) | < .005 |
| Creatinine 30 days post-transplantation (mg/dL)[b,c] | 2.31 ± 1.01 | 1.87 ± 1.07 | .614 |
| Immunosupression | | | |
| CI + sodium mycophenolate + steroids | 6 | 12 | .342 |
| Induction[d] + CI + sodium mycophenolate, steroids | 7 | 11 | .244 |

[a] Fisher's exact test Mean ± SD.

[b] Mean ± SD.Fisher's exact test

[c] Student's T Test

[d]Induction: 16 patients received Basiliximab® and two patients in the non-rejectors group received Thymoglobulin®; CI = calcineurin inhibitor (cyclosporine or tacrolimus)

**Table 2:** Diagnostic value of isolated and simultaneous TIM3 and Perforin gene expression measurements for the diagnosis of acute rejection of renal allografts.

| | | | | | Percentages | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Genes** | AUC | p-value[2] | Samples | Threshold [3] | Se | Sp | PPV | NPV | AC |
| **TIM 3** [1] | 0,752 | 0,000 | n=260 | 130% | 70 | 78 | 23 | 96 | 77 |
| **Perforin** [1] | 0,733 | 0,000 | n=260 | 94% | 78 | 66 | 18 | 96 | 66 |
| **Both +** [1] | | 0,000 | n=260 | Both | 60 | 84 | 27 | 96 | 82 |

[1] Positive scoring was determined from relative gene expression above (+) the gene and time-interval specific threshold values.

[2] Determined using Fisher's exact test.

[3] Values given are gene expression percentages relative to the postoperative set to 100%.

Se: sensitivity, Sp: specificity, PPV: positive predictive value, NPV: negative predictive value, AC: accuracy. Presented as percentages

**Legends to the figures.**

**Figure 1.** Receiver operating characteristic (ROC) curves generated to analyze the diagnostic parameters of mRNA gene expression. **Curve A, TIM 3** area under the curve (AUC) = 0.752 (95% CI: 0.653 – 0.852) **Curve B, Perforin** AUC 0.733 (95% CI 0.658 – 0.809).

**Figure 2: Pre and post acute rejection treatment gene expression analyzes. TIM3 and Perforin genes.** The box plot representation shows the medians and the percentile values 10, 25, 75 and 90 for the quantification levels of normalized mRNA ($2^{-\Delta\Delta Ct}$). **A:** TIM3 box plot of the gene expression pre-treatment and post-treatment ($p<0.001$). **B:** Perforin box plot of the gene expression pre-treatment and post-treatment ($p<0.001$).

**Figure 1.**

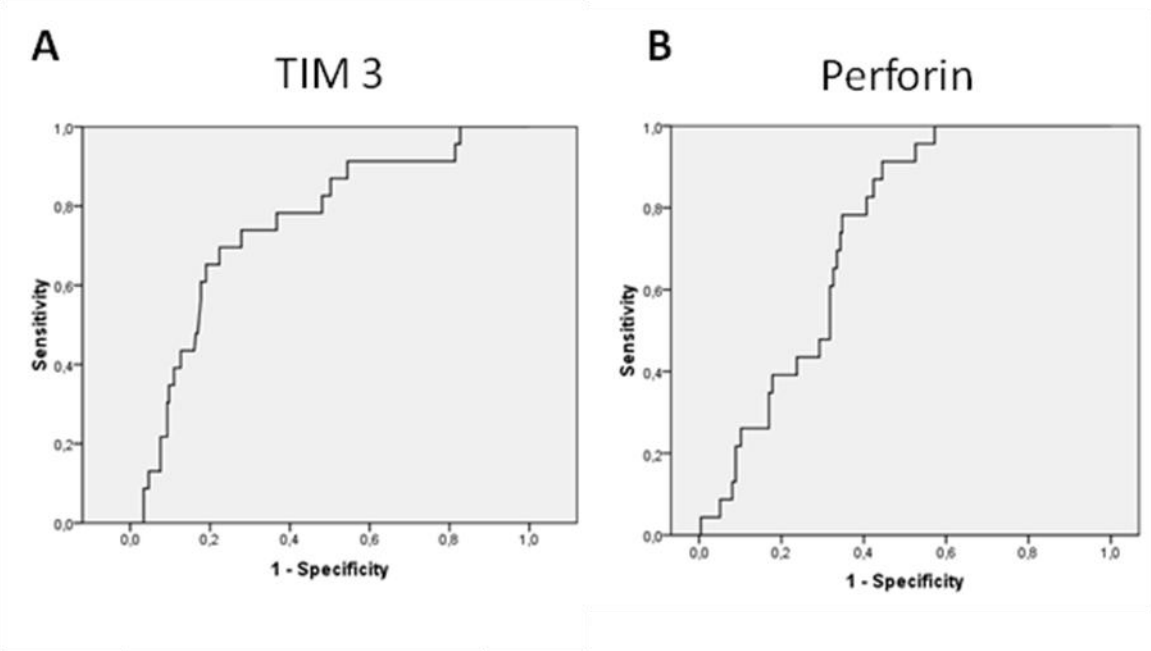
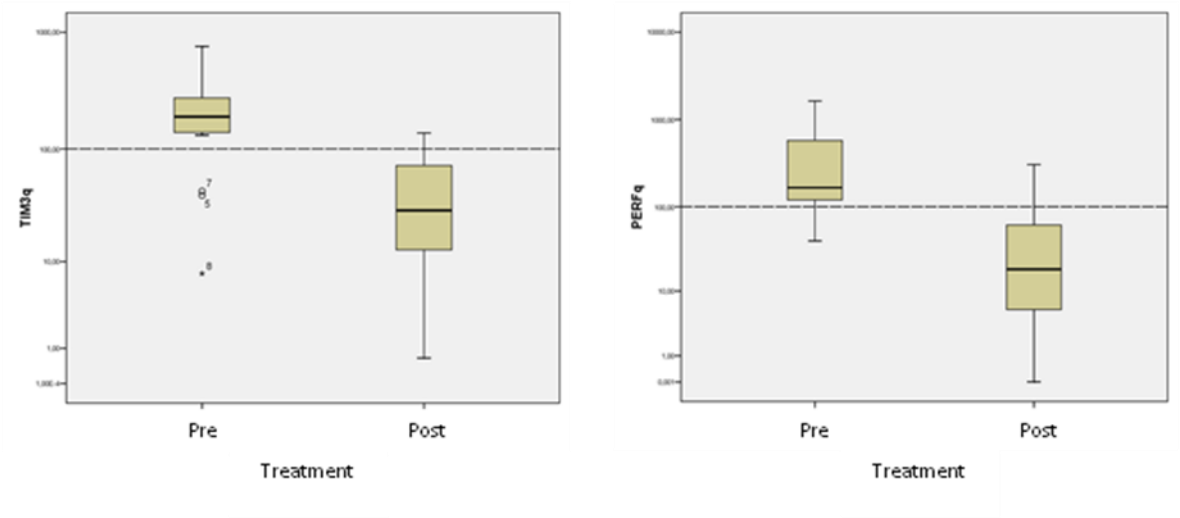

A
TIM 3
Sensitivity
1 - Specificity
B
Perforin
Sensitivity
1 - Specificity

**Figure 2.**



# Capítulo 7: Considerações Finais

As conclusões deste estudo serão apresentadas de acordo com os objetivos inicialmente propostos.

Em relação ao **objetivo geral** “Consolidar a mensuração do mRNA pela técnica de PCR em tempo real como instrumento para o diagnóstico não-invasivo da rejeição aguda de transplantes renais” conclui-se que ao final deste estudo, ainda faltam dados mais robustos de estudos envolvendo uma maior população de pacientes para que se abranja um número maior de patologias associadas ao período pós-transplante. Também se sugere que o período de avaliação dos pacientes seja estendido para que as patologias crônicas também possam ser avaliadas. Neste objetivo é, no entanto possível afirmar com segurança que os testes moleculares apresentem elevado valor preditivo negativo para o diagnóstico de rejeição aguda celular.

Em relação aos objetivos específicos, as considerações finais serão apresentadas a seguir:

**- Objetivo Específico 1.** Estudar longitudinalmente a cinética da expressão gênica pós-transplante renal imediato.

A utilização da técnica de quantificação relativa da expressão gênica para a avaliação da cinética da expressão gênica pós-transplante renal imediato se mostrou uma ferramenta útil, uma vez que de acordo com os resultados obtidos, os pacientes acometidos por episódios de rejeição aguda tiveram níveis de expressão elevados em relação a pacientes que não foram acometidos por este quadro. De elevada relevância, também encontramos que os valores preditivos negativos destas análises são muito elevados, ou seja, episódios de rejeição

dificilmente ocorrem na ausência de expressão gênica aumentada, no caso de quantificações diminuídas (testes negativos) pode-se excluir a probabilidade de o paciente ter a doença. Contrário a nossa hipótese, os valores preditivos positivos (VPP) foram menores do que o esperado, sugerindo que os VPPs de estudos transversais prévios possam ter sido super-estimados, pelo menos nos genes aqui avaliados. No único outro estudo longitudinal previamente publicado, os VPPs embora superiores aos nossos, já foram consideravelmente menores do que nos estudos transversais prévios. Suposições contextualizadas para os VPPs do presente estudo seriam: [1] Estudos de prevalência selecionam grupos de pacientes em situação clínica bem definida (por exemplo, rejeição aguda) e comparam com outros grupos igualmente em situações clínicas bem definidas, todas retrospectivamente diagnosticadas com base em critérios histológicos. Nessa abordagem, variações longitudinais da expressão gênica não são detectadas. Em contrapartida, quando são realizadas coletas seqüenciais de pacientes, variações da expressão são detectadas e quando as mesmas não estão relacionadas à ativação aloimune ou não são relevantes em termos de agressão do enxerto elas irão necessariamente reduzir os VPPs do teste. É entretanto possível que estas ativações sub-clínicas exerçam papel relevante na alorreatividade persistente relacionada à rejeição crônica. [2] Infecções virais intercorrentes, que podem ser silenciosas, também estão relacionadas com respostas do tipo Th1, que envolvem vários dos genes participantes da RA e da RASC. [3] O poder da ferramenta molecular. É plausível que a monitorização molecular detecte pequenos e possivelmente irrelevantes aumentos na expressão gênica que não cheguem a desencadear os mecanismos de agressão ao enxerto.

Em contrapartida, estes níveis sub-clínicos de expressão podem ser de fato prejudiciais aos enxertos promovendo o processo progressivo de fibrose. [4] Por fim pode-se argumentar que os baixos VPP são devidos a inadequação do padrão-ouro atualmente utilizado no transplante renal. Análises histológicas do tecido renal têm muitos pontos negativos, incluindo o fato do diagnóstico histológico ser arbitrário, menos sensível e tardio em relação à expressão gênica.

Entretanto, deve se considerar que a utilidade de um teste não se baseia apenas no seu VPP, mas que também os valores preditivos negativos (VPNs) são importantes. Desta maneira, podemos inferir que a abordagem molecular à injúria imune ao enxerto pode ser muito útil já que permite um diagnóstico acurado da ausência de rejeição, uma vez que uma quantificação negativa fornece uma probabilidade pós-teste de exclusão de rejeição superior à 95%.

**- Objetivo Específico 2.** Avaliar as modificações da quantificação gênica por RT-PCR em resposta aos tratamentos para a rejeição aguda de transplantes renais.

A quantificação de transcritos dos genes estudados mostrou o comportamento esperado, reduzindo significativamente seus níveis para menos de 50% relativos ao calibrador. Assim sendo, esta metodologia se credencia como ferramenta diagnóstica acurada em detectar a diminuição da expressão gênica em resposta ao tratamento imunossupressor dos casos de rejeição aguda de enxertos renais.

**- Objetivo Específico 3.** Identificar entre os genes candidatos os que apresentam melhores parâmetros para a monitorização da imunossupressão em pacientes transplantados renais.

Dentre os genes estudados, TIM3 e Perforina, pelos resultados demonstrados, o TIM3 parece ser um biomarcador mais promissor e fidedigno da resposta imune ao órgão transplantado. Entretanto, fica evidente que a avaliação combinada de um grupo de genes parece ser mais acurada nas abordagens moleculares. A análise de um conjunto de genes relacionados com o processo inflamatório de agressão ao enxerto somadas a genes relacionados a patologias associadas, tais como fibrose, NTA, infecções virais, entre outros, parece ser a maneira correta de proceder para que este tipo de ferramenta diagnóstica venha a ser implantada na prática clínica.

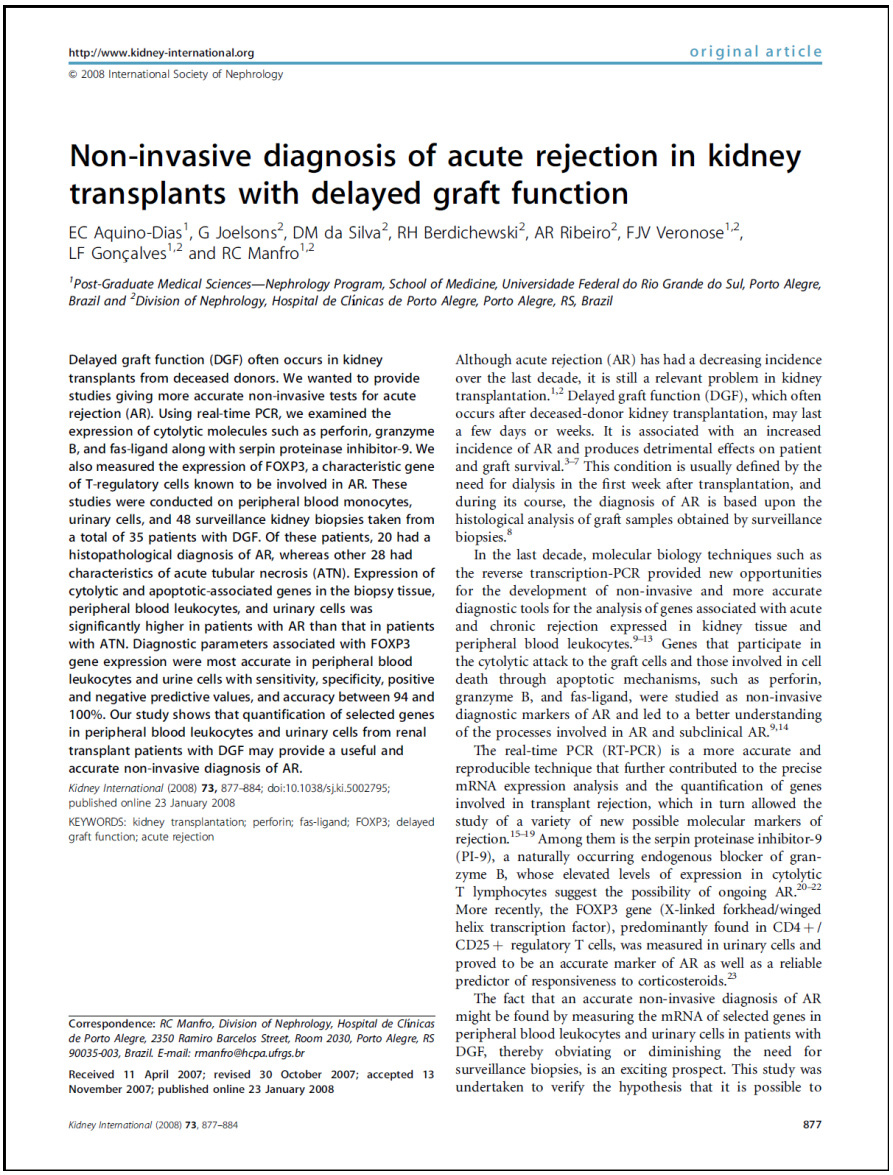
Por fim considera-se que um longo caminho ainda deva ser percorrido antes que qualquer ensaio molecular possa ser considerado adequado como teste diagnóstico em nível clínico. Especificamente o teste deve ser: [1] avaliado em mais de uma coorte; [2] avaliado em uma série de pacientes consecutivos com múltiplas patologias para permitir a verdadeira determinação de seus parâmetros diagnósticos; [3] reproduzido por grupos independentes; [4] avaliado longitudinalmente para determinar a habilidade de um dado ensaio de detectar processos patológicos precocemente e tardiamente pós-transplante; [5] demonstrar que o teste candidato retorna aos valores basais com terapia efetiva. Além de satisfazer esses critérios básicos, um teste útil também deve ser capaz de detectar níveis sub-clínicos de agressão para que a intervenção precoce seja uma opção potencial (13).

# Capítulo 8: Anexos

# 8.1. Produção Científica no Período

## 8.1.1. Non-invasive diagnosis of acute rejection in kidney transplants with delayed graft function

Aquino-Dias EC, Joelsons G, Silva DM, Berdichewski RH, Ribeiro AR, Veronese FJV, Gonçalves LF, Manfro RC. Non-invasive diagnosis of acute rejection in kidney transplants with delayed graft function. Kidney Int 2008; 73: 877.



http://www.kidney-international.org original article



### Non-invasive diagnosis of acute rejection in kidney transplants with delayed graft function

EC Aquino-Dias[1], G Joelsons[2], DM da Silva[2], RH Berdichewski[2], AR Ribeiro[2], FJV Veronose[1,2], LF Gonçalves[1,2] and RC Manfro[1,2]

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**Delayed graft function (DGF) often occurs in kidney transplants from deceased donors. We wanted to provide studies giving more accurate non-invasive tests for acute rejection (AR). Using real-time PCR, we examined the expression of cytolytic molecules such as perforin, granzyme B, and fas-ligand along with serpin proteinase inhibitor-9. We also measured the expression of FOXP3, a characteristic gene of T-regulatory cells known to be involved in AR. These studies were conducted on peripheral blood monocytes, urinary cells, and 48 surveillance kidney biopsies taken from a total of 35 patients with DGF. Of these patients, 20 had a histopathological diagnosis of AR, whereas other 28 had characteristics of acute tubular necrosis (ATN). Expression of cytolytic and apoptotic-associated genes in the biopsy tissue, peripheral blood leukocytes, and urinary cells was significantly higher in patients with AR than that in patients with ATN. Diagnostic parameters associated with FOXP3 gene expression were most accurate in peripheral blood leukocytes and urine cells with sensitivity, specificity, positive and negative predictive values, and accuracy between 94 and 100%. Our study shows that quantification of selected genes in peripheral blood leukocytes and urinary cells from renal transplant patients with DGF may provide a useful and accurate non-invasive diagnosis of AR.**

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KEYWORDS: kidney transplantation; perforin; fas-ligand; FOXP3; delayed graft function; acute rejection

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Although acute rejection (AR) has had a decreasing incidence over the last decade, it is still a relevant problem in kidney transplantation.[1,2] Delayed graft function (DGF), which often occurs after deceased-donor kidney transplantation, may last a few days or weeks. It is associated with an increased incidence of AR and produces detrimental effects on patient and graft survival.[3–7] This condition is usually defined by the need for dialysis in the first week after transplantation, and during its course, the diagnosis of AR is based upon the histological analysis of graft samples obtained by surveillance biopsies.[8]

In the last decade, molecular biology techniques such as the reverse transcription-PCR provided new opportunities for the development of non-invasive and more accurate diagnostic tools for the analysis of genes associated with acute and chronic rejection expressed in kidney tissue and peripheral blood leukocytes.[9–13] Genes that participate in the cytolytic attack to the graft cells and those involved in cell death through apoptotic mechanisms, such as perforin, granzyme B, and fas-ligand, were studied as non-invasive diagnostic markers of AR and led to a better understanding of the processes involved in AR and subclinical AR.[9,14]

The real-time PCR (RT-PCR) is a more accurate and reproducible technique that further contributed to the precise mRNA expression analysis and the quantification of genes involved in transplant rejection, which in turn allowed the study of a variety of new possible molecular markers of rejection.[15–19] Among them is the serpin proteinase inhibitor-9 (PI-9), a naturally occurring endogenous blocker of granzyme B, whose elevated levels of expression in cytolytic T lymphocytes suggest the possibility of ongoing AR.[20–22] More recently, the FOXP3 gene (X-linked forkhead/winged helix transcription factor), predominantly found in CD4+/CD25+ regulatory T cells, was measured in urinary cells and proved to be an accurate marker of AR as well as a reliable predictor of responsiveness to corticosteroids.[23]

The fact that an accurate non-invasive diagnosis of AR might be found by measuring the mRNA of selected genes in peripheral blood leukocytes and urinary cells in patients with DGF, thereby obviating or diminishing the need for surveillance biopsies, is an exciting prospect. This study was undertaken to verify the hypothesis that it is possible to

diagnose AR in kidneys with DGF based on mRNA quantification, in urine and/or peripheral blood, of genes that act as markers or that are mechanistically involved in the AR process of kidney allografts.

## RESULTS

From January 2004 to October 2006, 165 patients received kidney transplants in our unit. The 35 patients who had DGF for a period of at least 7 days and who had undergone a graft biopsy during the DGF period, as well as 30 comparator patients, were included in the study. Table 1 shows the main demographic data of the groups. Sampling was predominantly constituted of biological materials obtained from kidney transplants harvested from deceased donors, with a higher proportion of men in all groups. No statistically significant differences were observed between the groups with reference to age, gender, and donor type distributions (analysis of variance, Dunnett's test, $P>0.05$). There were no statistically significant differences in time intervals between the transplant and the moment of biopsy in the group of patients with acute tubular necrosis (ATN) and the comparator group with biopsies considered as normal (NOR), as compared to the group with histopathological diagnosis of AR (analysis of variance, Dunnett's test, $P>0.05$). The mean time intervals in the group with calcineurin inhibitor-induced nephrotoxicity (CIN) and in the group with chronic allograft nephropathy (CAN) were significantly higher than that in the AR group (analysis of variance, Dunnett's test, $P<0.001$). The mean time intervals after the transplant in patients of the NOR group were significantly lower than those of the CIN group ($P=0.015$) and CAN group ($P<0.001$) (Table 1).

Forty-eight surveillance biopsies were collected from 35 patients with DGF, 20 of which presented with histopathological features of AR and 28 with histopathological diagnosis of ATN. The time for the diagnosis of AR ranged from 7 to 55 days (mean $21.4\pm15.6$ days, median 14.5 days). According to the Banff classification,[24] three cases were classified as borderline, eight as type IA, six as type IIA, and three as type III. C4d deposition in peritubular capillaries was present in one case. Serum creatinine levels did not differentiate AR from ATN ($5.32\pm3.36$ versus $5.96\pm2.01$ mg per 100 ml, respectively; $P=0.784$). Creatinine levels in the comparator groups were as follows: CIN: $3.08\pm1.16$ mg per 100 ml; CAN: $4.65\pm1.95$ mg per 100 ml; and NOR: $1.69\pm0.4$ mg per 100 ml. The group of patients with normal biopsies had a lower mean serum creatinine than the groups with AR ($P<0.001$), ATN ($P<0.001$), and CAN ($P=0.014$). No statistically significant differences were observed in the comparison of the mean serum creatinine between the NOR and the CIN groups ($P=0.625$).

Perforin, granzyme B, and fas-ligand gene expression levels in kidney tissue, peripheral blood leukocytes, and urinary cells are shown in the box plots presented in Figure 1. Similarly, FOXP3 and PI-9 results are displayed in Figure 2. For all genes analyzed, significantly higher quantities of transcripts were present in AR than that in ATN. These findings were observed in kidney tissue, peripheral blood leukocytes, and urinary cells. Similarly, in all compartments, the normalized quantities of the studied genes were significantly higher in samples of patients with DGF and AR than that of patients of the comparator groups. The only exception occurred with the PI-9 gene quantification, in which the amount observed in AR did not differ from the amount present in the group of patients with CIN (Figure 2).

Table 2 shows Spearman's correlation coefficients between the genes in the different compartments. Strong and significant correlations were observed between the quantifications in tissue, blood, and urine for all genes. Although all correlations reached statistical significance, those observed for the FOXP3 gene were the strongest, with correlation coefficient values of 0.95 or higher.

The areas under the curves and cutoff points obtained from receiver-operating characteristic curve analyses, and the

**Table 1 | Demographic data of studied groups and comparators**

| Groups (number of patients) | DGF (35) | | Comparators (30) | | |
|---|---|---|---|---|---|
| Histopathological diagnoses (number of samples) | AR (20) | ATN (28) | CIN (8) | CAN (12) | NOR (10) |
| Age (years; mean ± s.d.)[a] | 41 ± 13 | 47 ± 10 | 43 ± 11 | 47 ± 12 | 47 ± 08 |
| Gender (male/female)[b] | 8/6 | 15/6 | 03/05 | 07/05 | 07/03 |
| Transplant number (1st, 2nd) | 14/0 | 20/1 | 6/2 | 9/3 | 10/0 |
| Donor (living/deceased)[b] | 3/11 | 2/19 | 01/07 | 01/11 | 01/09 |
| Cold ischemia time (hours; mean ± s.d.)[a] | 14.4 ± 8.5 | 16.8 ± 6.6 | 12.4 ± 8.8 | 16.9 ± 7.2 | 16.8 ± 7.8 |
| Time to biopsy (days; mean ± s.d.)[a] | 21 ± 15 | 21 ± 14 | 225 ± 212[c] | 269 ± 213[c] | 74 ± 8[d,e] |
| Donors' CMV status (positive/negative)[b] | 14/0 | 20/1 | 8/0 | 12/0 | 10/0 |
| Recipients' CMV status (positive/negative)[b] | 13/1 | 20/1 | 8/0 | 12/0 | 9/1 |
| Panel reactive antibodies (%; mean ± s.d.)[a] | 19.4 ± 33.6 | 2.20 ± 11.0[f] | 0 ± 0 | 15.6 ± 26.9 | 8.4 ± 22.6[g] |
| HLA mismatches (A, B, DR; mean ± s.d.)[a] | 3.07 ± 1.82 | 3.35 ± 1.27 | 3.75 ± 1.26 | 4.29 ± 1.38 | 2.88 ± 1.35 |
| Serum creatinine (mg per 100 ml; mean ± s.d.)[a] | 5.32 ± 3.36 | 5.96 ± 2.01 | 3.08 ± 1.16 | 4.65 ± 1.95 | 1.69 ± 0.4[c,h] |
| Induction (Thymoglobulin/Basiliximab) | 3/11 | 0/21 | 0/4 | 2/6 | 1/6 |

ANOVA, analyses of variance; AR, acute rejection; ATN, acute tubular necrosis; CAN, chronic allograft nephropathy; CIN, calcineurin inhibitor-induced nephrotoxicity; CMV, cytomegalovirus; DGF, delayed graft function; NOR, normal; s.d., standard deviation.
[a]ANOVA with Dunnett's test; [b]Fisher's exact test; [c]$P<0.001$ versus AR and ATN; [d]$P<0.02$ versus CIN; [e]$P<0.001$ versus CAN; [f]$P<0.01$ versus AR; [g]$P<0.05$ versus AR; [h]$P<0.02$ versus CAN.
None of the other comparisons showed statistically significant differences.

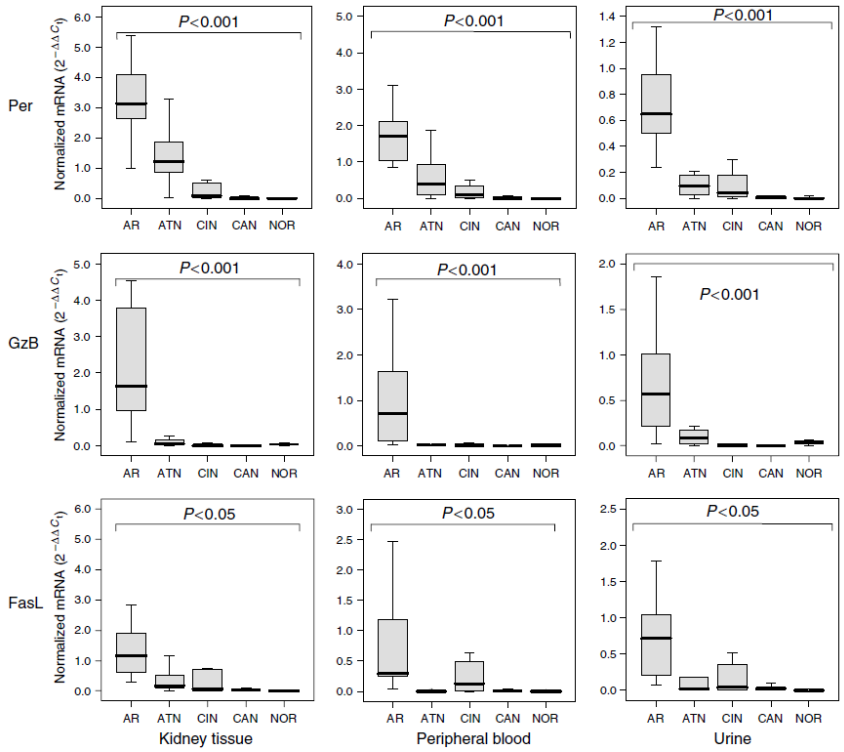


**Figure 1 | mRNA levels of perforin, granzyme B, and fas-ligand in kidney tissue, peripheral blood leukocytes, and urinary cells according to the histological diagnosis.** The box-plot representation shows the medians and the percentile values 10, 25, 75, and 90 for the quantification levels of normalized mRNA ($2^{-\Delta\Delta C_T}$). β-Actin quantification was used for normalizing the mRNA amounts present in kidney tissue and peripheral blood leukocytes. Cyclophilin quantification was used for mRNA normalization in urinary cells. The expression levels of Per, GzB, and FasL genes were significantly higher in patients with AR as compared to patients with ATN and to the control groups: CIN, CAN, and NOR in kidney tissue, peripheral blood, and urinary cells. *P*-values are the lowest of the comparisons between each compartment per gene. Per, perforin; GzB, granzyme B; FasL, fas-ligand; AR, acute rejection; ATN, acute tubular necrosis; CIN, calcineurin inhibitor-induced nephrotoxicity; CAN, chronic graft nephropathy; and NOR, normal.

diagnostic parameters for AR of the gene quantifications in peripheral blood leukocytes and urinary cells are all presented in Tables 3 and 4, respectively. In peripheral blood, all genes showed a good diagnostic performance. However, the FOXP3 gene analysis showed higher accuracy than the other genes evaluated (Table 3). In the analysis of diagnostic parameters derived from gene quantification in urinary cells, again the FOXP3 gene was the most precise, showing absolute accuracy, whereas the PI-9 gene showed lower performance, mainly because of its low specificity and positive predictive values.

## DISCUSSION

In studies that did not include patients with DGF, increased amounts of gene transcripts of cytotoxic molecules, PI-9 and FOXP3 have been found in mRNA obtained from kidney tissue, peripheral blood cells, and urinary cells of renal transplant patients with acute graft rejection.[10–12,25] The better standardization and reproducibility of the RT-PCR method allowed for a significant progress in gene quantitative transcriptional profiling. By using this technique, it is possible to monitor intragraft inflammatory events and detect AR episodes,[15,17,26] determine their diagnosis in advance,[21,27] predict treatment responsiveness,[18] and differentiate them from urinary tract infections.[28] However, the usefulness of this method has not been properly evaluated during DGF, a situation in which a non-invasive method of adequate accuracy would be a cost-effective aid to clinical management.

In this study, evaluating patients with DGF, mRNA quantification by RT-PCR showed that the studied genes showed increased expression in AR in all compartments

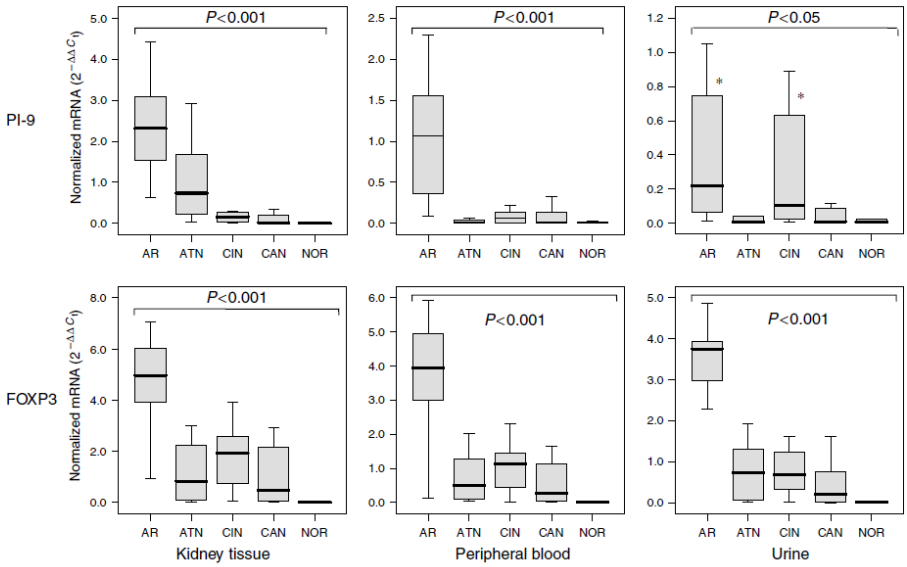


**Figure 2 | mRNA levels of PI-9 and FOXP3 in kidney tissue, peripheral blood leukocytes, and urinary cells according to the histological diagnosis.** The box-plot representation shows the medians and the percentile values 10, 25, 75, and 90 for the quantification levels of normalized mRNA ($(2^{-\Delta\Delta C_T})$. β-Actin quantification was used for normalizing the mRNA amounts present in kidney tissue and peripheral blood leukocytes. Cyclophilin quantification was used for mRNA normalization in urinary cells. The expression level of PI-9 was higher in patients with AR as compared to patients with ATN and to the control groups CAN and NOR in the three compartments. *P*-values are the lowest in comparisons in each compartment. *There was no significant difference in PI-9 gene quantification in urinary cells when comparing the AR and CIN groups ($P > 0.05$). The levels of FOXP3 were always higher in AR cases as compared to all other groups. PI-9 = serpin proteinase inhibitor-9; FOXP3 = X-linked forkhead/winged helix transcription factor-3.

**Table 2 | Spearman's correlation coefficients of gene quantifications at the different compartments**

| | Compartments | | | | | |
|---|---|---|---|---|---|---|
| | Peripheral blood-tissue | | Urinary cells-tissue | | Urinary cells-peripheral blood | |
| Gene | Correlation coefficient | *P*-value | Correlation coefficient | *P*-value | Correlation coefficient | *P*-value |
| Per | 0.939 | <0.001 | 0.885 | <0.001 | 0.929 | <0.001 |
| GzB | 0.822 | <0.001 | 0.883 | <0.001 | 0.825 | <0.001 |
| FasL | 0.601 | <0.001 | 0.668 | <0.001 | 0.842 | <0.001 |
| PI-9 | 0.767 | <0.001 | 0.540 | <0.001 | 0.707 | <0.001 |
| FOXP3 | 0.976 | <0.001 | 0.980 | <0.001 | 0.950 | <0.001 |

FasL, fas ligand; FOXP3, X-linked forkhead/winged helix transcription factor-3; GzB, granzyme B; Per, perforin; PI-9, serpin proteinase inhibitor-9.

studied. In the evaluation of the mRNA from peripheral blood leukocytes, the analysis of diagnostic parameters showed that all genes produced accurate parameters. However, FOXP3 produced the best diagnostic parameters by having higher specificity, positive predictive value, and overall accuracy. FOXP3 mRNA in peripheral blood leukocytes has been shown to correlate with the amount of CD4+/CD25+ T cells in peripheral blood of kidney transplant patients.[29] During AR, elevated FOXP3 mRNA within the graft, in urinary cells, and in peripheral blood cells suggests the development of a host immune regulatory graft protective response. It is conceivable that the increased amount of FOXP3 mRNA found in patients with rejecting grafts mirrors an active response to graft-damaging effector T cells, showing that the immune response to the graft cells possesses both cyto-destructive and cyto-protective components.[29,30] Alternatively, as suggested in recent studies with human cells, it is also possible that the elevated levels of FOXP3 mRNA found in the group of patients with rejection represent an activation-induced phenomenon that might not be involved in regulatory activities of the immune response.[31,32]

**Table 3 | Cutoff points and diagnostic parameters, in percentage, of molecular quantifications to the AR diagnosis in peripheral blood of patients with DGF**

| Gene | AUC (CI 95%) | Cutoff point | Se | Sp | PPV | NPV | A |
|---|---|---|---|---|---|---|---|
| Per | 0.881 (0.766-0.966) | 0.85 | 100 | 75 | 77 | 100 | 86 |
| GzB | 0.941 (0.869–1.013) | 0.05 | 88 | 90 | 88 | 90 | 89 |
| FasL | 0.935 (0.852–1.019) | 0.15 | 89 | 94 | 90 | 95 | 92 |
| PI-9 | 0.935 (0.850–1.021) | 0.20 | 88 | 90 | 88 | 90 | 88 |
| FOXP3 | 0.954 (0.871–1.037) | 1.85 | 94 | 95 | 94 | 95 | 95 |

95% CI, 95% confidence interval; A=accuracy (% of cases correctly classified); AR, acute rejection; AUC, area under the curve; DGF, delayed graft function; FasL, fas ligand; FOXP3, X-linked forkhead/winged helix transcription factor-3; GzB, granzyme B; NPV, negative predictive value; Per, perforin; PI-9, serpin proteinase inhibitor-9; PPV, positive predictive value; Se, sensitivity; Sp, specificity.

**Table 4 | Cutoff points and diagnostic parameters, in percentage, of molecular quantifications for the AR diagnosis in urinary cells of patients with early graft function**

| Gene | AUC (CI 95%) | Cutoff point | Se | Sp | PPV | NPV | A |
|---|---|---|---|---|---|---|---|
| Per | 0.917 (0.788–1.046) | 0.22 | 100 | 86 | 86 | 100 | 92 |
| GzB | 0.851 (0.692–1.011) | 0.20 | 83 | 86 | 83 | 86 | 85 |
| FasL | 0.887 (0.763–1.011) | 0.07 | 100 | 64 | 71 | 100 | 81 |
| PI-9 | 0.875 (0.745–1.005) | 0.024 | 92 | 64 | 67 | 90 | 76 |
| FOXP3 | 1.000 (1.000–1.000) | 2.2 | 100 | 100 | 100 | 100 | 100 |

95% CI, 95% confidence interval; A, accuracy (% of cases correctly classified); AR, acute rejection; AUC, area under the curve; DGF, delayed graft function; FasL, fas ligand; FOXP3, X-linked forkhead/winged helix transcription factor-3; GzB, granzyme B; NPV, negative predictive value; Per, perforin; PI-9, serpin proteinase inhibitor-9; PPV, positive predictive value; Se, sensitivity; Sp, specificity.

The evaluation of mRNA levels in peripheral blood leukocytes of patients with kidney transplants can be a valuable tool for the diagnosis of rejection, as these analyses do not depend on the urine production by the graft. In previous studies in which mRNA was quantified by reverse transcription competitive PCR, Vasconcellos *et al.*[12] demonstrated that in patients without DGF, the mRNA evaluation of peripheral blood may produce proper diagnostic parameters for AR.

The mRNA analyses of urine-sediment cells of kidney transplant patients are interesting, given that under most circumstances the lymphocytes present in urine have trafficked through the kidney, and therefore the status of these cells might accurately reflect the status of graft-infiltrating lymphocytes. However, this will depend on the urine production by the graft, which can be a limitation in anuric ATN. Another limitation is the possibility of analyzing urine produced by the native kidney and not by the graft. In this study, it was not possible to have a proper urine sample for analysis in 20% of the cases, but when urine was available, accurate diagnostic parameters were achievable. The evaluations of mRNA amounts amplified from urinary cells resulted in diagnostic parameters that were similar to those found in the peripheral blood evaluation. Interestingly, the analysis of FOXP3 mRNA amounts in the urinary cells allowed for perfect diagnostic accuracy. In the FOXP3 mRNA analysis, our data of patients with DGF were comparable to the description found in a recent study by Muthukumar *et al.*,[23] where the levels of FOXP3 and perforin in urinary cells were significantly higher in patients with AR, as compared to groups with histological diagnoses of chronic graft nephropathy and transplants with stable function.

In the analyses of both peripheral blood and urinary cells, the molecular mRNA quantifications allowed for the differentiation of AR from other clinical situations. Such differentiations had already been shown in urinary cells in other studies.[21,23,27,33,34] However, evaluations in patients with DGF are quite limited. Only two studies reported this evaluation in a rather restricted number of patients. By using the competitive PCR method, Li *et al.*[25] observed that in 9 out of 11 patients with DGF, the perforin and granzyme B expression levels were significantly higher in patients with AR. Using the RT-PCR technique, Yannaraki *et al.*[35] evaluated five patients with DGF and reported that the detected amounts of perforin, granzyme B, and fas-ligand were also increased in patients with AR.

Amounts of mRNA of other genes have been evaluated for the diagnosis of AR. Urinary levels of CD103 mRNA were described by Ding *et al.*[27] Granulysin expression showed a strong predictive value for rejection episodes early after kidney transplantation.[17] The chemokine interferon-inducible protein 10 mRNA expression analysis allowed for the differentiation between urinary tract infections and cytomegalovirus infections and AR.[34] Evaluation of interleukin-4 and tumor necrosis factor-$\alpha$ genes revealed the possibility of diagnosing rejection before the appearance of its clinical and laboratorial signs.[36] The analyses of the above-mentioned genes are certainly worth performing in the DGF setting.

However, two recent studies did not find mRNA evaluations to be useful for the diagnosis of AR in renal transplantation.[35,37] In the first study, Yannaraki *et al.*[35] evaluated the expression of the perforin, granzyme B, and fas-ligand genes and demonstrated that their levels were increased in AR, but a similar profile was also present in other clinical situations, such as cytomegalovirus infections, urinary infections, and DGF, the latter having been evaluated in a restricted number of patients. The other study, using a conventional semiquantitative PCR technique, suggested that the expression of perforin and fas-ligand genes in kidney tissue could predict the diagnosis of AR, but the expression of these genes in peripheral blood did not correlate with the histopathological diagnosis of AR. The discrepancy between these results and many others in the literature, including ours, is probably related to methodological issues such as sample size and factors related to urine sampling that might have affected other stages of the PCR. Recommendations for the collection and storage of urinary cells were proposed by Medeiros *et al.*,[38] suggesting the importance of the utilization of an RNA stabilization solution.

As limitations of this study, it must be acknowledged that the samples of patients with CIN, CAN, and normal transplant kidneys were not obtained from patients with

DGF. Although chronic graft nephropathy is not an expected diagnosis during DGF, CIN may occur early after transplantation and could play an important role in delaying functional graft recovery.[4] It is also important to emphasize that a more appropriate control group would have been found by obtaining biopsies, blood, and urine from patients without DGF at time points similar to those obtained from patients with DGF. However, obtaining early biopsies from well-functioning grafts during the first post-transplant month is not an established practice and may raise ethical issues due to the risks imposed by doing it.

The findings of this study allowed for the conclusion that the analysis of mRNA quantifications of the genes involved in the alloimmune response in patients with DGF, both from peripheral blood leukocytes and from urine-sediment cells, provides an accurate molecular signature for the diagnosis of AR. The validation of these results in a multicenter, longitudinal study and possible sophistications of employed techniques may enable the molecular evaluations to replace or reduce the need for surveillance biopsies in kidney recipients during the DGF period. Besides, the combined analyses of different genes, perhaps in different compartments, may lead to even more precise diagnoses in this difficult clinical situation.

## MATERIALS AND METHODS

### Patients and sample collection

Primary renal disorders leading to end-stage renal failure were as follows: hypertensive glomerulosclerosis in 14 (8 with DGF) patients; chronic glomerulonephritis in 10 (6 with DGF) patients; obstructive uropathy and chronic pyelonephritis in 7 (3 with DGF) patients; diabetic nephropathy, adult polycystic kidney disease, and systemic lupus erythematosus in 5 (4, 3, and 2 with DGF, respectively) patients for each condition; and undetermined in 19 (9 with DGF) patients.

Patients with transplanted kidneys with DGF, defined by the need for dialysis during the first week after transplantation, were submitted to surveillance biopsies every 7–10 days until kidney function was recovered or graft was lost. Vascular, urological, or infectious reasons for graft dysfunction were ruled out by Doppler ultrasound, nuclear scans, and urine cultures before the biopsies were performed. Immediately before each biopsy, peripheral blood and sterile urine samples were collected from patients with diuresis. Two core fragments were obtained in the biopsies. The kidney biopsies were performed through ultrasonography guidance, using a semi-automatic gun with a 16-G needle. One and a half fragments were used for histological analyses and half a fragment was immediately frozen in liquid nitrogen and maintained at −70 °C. Immediately after the collection, the blood and urinary cells were isolated and frozen for later RNA extraction. Slide evaluation was performed by a kidney pathologist who was unaware of the clinical data. The Banff-97 classification was used for the histopathological diagnoses.[19] Patients with DGF were classified as having either ATN or AR. Patients with histopathological diagnoses of CIN ($n=8$), chronic graft nephropathy (CAN; $n=12$), and normal transplanted kidney (NOR; $n=10$) were used as comparators. Patients in the comparator groups were randomly selected among patients who had biopsies performed to elucidate graft dysfunction or as protocol biopsies, and they all had blood and urine collection at the time of their biopsies.

In the DGF group, corticosteroids combined with cyclosporin or tacrolimus and mycophenolate (mofetil or sodium) were used as immunosuppressive therapy for all patients. Induction with polyclonal antilymphocyte antibodies (Thymoglobulin) was performed in three patients considered as having high immunological risk, whereas all the other patients in the DGF group received interleukin-2 anti-receptor antibodies (Basiliximab). Among the comparators, 11 patients received triple therapy without antibody induction, three received antilymphocyte antibodies (Thymoglobulin), and 16 were induced with interleukin-2 anti-receptor antibodies (Basiliximab).

The study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre, accredited by the National Research Council of the Brazilian Ministry of Health, and registered at the Office for Human Research Protections—OHRP-USDHHS (Institutional Review Board—IRB 00000921).

### RNA extraction

Graft fragments were defrosted, macerated, and processed for the RNA extraction, using the QIAamp RNA Blood mini kit (Qiagen Inc., Chatsworth, CA, USA) method, according to the manufacturer's instructions. Peripheral blood was collected in tubes containing EDTA and the cells were separated with an erythrocyte lysis buffer. Urinary cells were obtained by centrifugation at 500 *g* for 20 min. The floating material was discarded and cell pellets were resuspended in a phosphate-buffered saline solution, centrifuged again for 10 min, and stored at −70 °C. RNA was extracted from cell pellets and isolated from peripheral blood and urine-sediment cells by using the same method as for the renal tissue. RNA quality was assessed by evaluating the optical density (OD) 260/280 ratio, and only RNA samples with OD ratio higher than 1.7 were analyzed, provided that a sufficient amount was available. In 10 occasions (21%), urine samples were either not available (six occasions, 12.5%) or did not provide enough RNA for analyses (four occasions, 8.3%).

### mRNA quantification

The expression of each gene was analyzed by using the relative quantification technique through RT-PCR employing the ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) detection system. Gene expression assays consist of a mixture of primers and TaqMan MGB (minor groove binding) probes at 360 μM concentrated 20 times. Primers were designed based upon the identification of target sequences at the gene bank. The sequences utilized had already been designed, tested, and previously validated by the manufacturer, and all primers utilized are intron-spanning to avoid genomic DNA amplification (Gene Expression Assays/Custom primers and probes; Applied Biosystems). The fluorescent dyes used as markers of the probes were 6-carboxyfluorescein (FAM) as reporter (at 5′) and 6-carboxytetramethyl rhodamine (TAMRA) as quencher (at 3′). The following genes were analyzed: perforin (Pf; ID: 65328531A; GenBank reference: 5551), granzyme B (GB; ID: Hs 00188051_m1; GenBank reference: 3002), fas-ligand (FasL; ID: Hs 00181225_m1; GenBank reference: 356), PI-9 (ID: Hs 00244603_m1; GenBank reference: 5272), and FOXP3 (ID: Hs 00203958_m1; GenBank reference: 50943). The endogenous molecular controls used were β-actin (PN 4310881E) for biopsy and peripheral blood samples, and cyclophilin (PN 4310883E) for urine samples (TaqMan PDAR Endogenous Control). To have equivalent amounts of mRNA in each reaction, sample normalization was achieved by using the controls described above.

The reactions were made in duplicates using the TaqMan EZ RT-PCR (PN N808-0235) (Applied Biosystems) kit, according to the following protocol: 5.0 µl of 5 × TaqMan EZ buffer, 3.0 µl of manganese acetate (25 mM), 0.75 µl of dATP (10 mM), 0.75 µl of dCTP (10 mM), 0.75 µl of dGTP (10 mM), 0.75 µl of dUTP (20 mM), 1.0 µl of recombinant *Thermus thermophilus* DNA polymerase (2.5 U/µl), 0.25 µl of AmpErase UNG (1 U/µl), and pure water to reach a volume of 23 µl. For each reaction, 1 µl of primers and probes (20 × ) and 1 µl of RNA were added to this mixture, reaching a final volume of 25 µl. The cycling program consisted of heating at 50 °C for 2 min, 60 °C for 30 min, followed by heating to 95 °C for 5 min and 40 cycles using the temperatures of 94 °C for 20 s and 62 °C for 60 s. The intrasample variability of all the RT-PCR performed was always lower than 1%. For all duplicates analyzed, the mean intrasample variability percentage was 0.038 ± 0.02.

In the DGF group, 28 patients had one set of analyses, three patients had two sets, two patients had three sets, and two patients had four sets of analyses. The analyses of amplified products were performed through the relative quantification method ($2^{-\Delta\Delta C_T}$, which describes alterations to the target gene expression concerning a reference sample.[39]

### Statistical analyses

Data are presented as absolute numbers, mean ± s.d., or percentages. Gene quantifications are shown as box-plot representations. Continuous variables with normal distributions were evaluated using the analysis of variance followed by the Dunnett's test. The mRNA levels were analyzed using the Kruskal–Wallis test with all five diagnostic groups. Dunn's test was used for multiple comparisons among the various groups. Mann–Whitney's test was used for comparisons between two groups. Fisher's exact test was used for the analyses of categorical variables. The correlations between the mRNA levels with all genes were calculated by using Spearman's correlation test. Receiver-operating characteristic curves were generated to find the best cutoff points for the diagnoses of AR.[40] All analyses were performed by using the SPSS (Statistical Package for the Social Sciences) program (version 14.0; SPSS Inc., Chicago, IL, USA). The *P*-values were adjusted by using Finner's method, and the statistical significance level was established as $P < 0.05$.

### ACKNOWLEDGMENTS

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## 8.1.2. Noninvasive Tim-3 Messenger RNA Evaluation in Renal Transplant Recipients with Graft Dysfunction

Manfro RC, Aquino-Dias EC, Joelsons G, Nogare AL, Carpio VN, Gonçalves LF. Noninvasive Tim-3 messenger RNA evaluation in renal transplant recipients with graft dysfunction. Transplantation 2008; 86: 1869-1874.

# Noninvasive Tim-3 Messenger RNA Evaluation in Renal Transplant Recipients With Graft Dysfunction

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**Background.** Renal biopsies are usually needed to elucidate graft dysfunction. In this study, T-cell immunoglobulin domain, mucin domain mRNA expression in the peripheral blood leukocytes (PBL) and urinary cells (UC) were studied as a noninvasive method for the diagnosis of acute rejection (AR) of kidney transplant patients with dysfunction.

**Methods.** One hundred sixty biopsies were obtained from 115 patients. Blood and urine samples were collected immediately before the biopsies. Histopathologic diagnoses were acute tubular necrosis with superimposed AR or acute tubular necrosis in patients with delayed graft function (DGF), and (AR), or calcineurin inhibitor nephrotoxicity (CIN), or interstitial fibrosis and tubular atrophy in patients with acute graft dysfunction (AGD). Fifteen protocol biopsies of stable grafts were used as controls. mRNA relative quantification was performed by real-time polymerase chain reaction.

**Results.** Gene expression in tissue, PBL, and UC was always higher in patients with AR than in patients with the other causes of graft dysfunction ($P<0.001$). Significant correlations of gene expression in different compartments were observed ($P<0.001$). The obtained diagnostic parameters were 100% accurate in the DGF group and, respectively, for blood and urine: sensitivity (87% and 84%); specificity (95% and 96%); positive predictive value (87% and 89%); negative predictive value (93% and 94%); and accuracy (91% and 93%) for the group of patients with AGD.

**Conclusion.** T-cell immunoglobulin domain, mucin domain mRNA quantification by real-time polymerase chain reaction in PBL and UC of renal transplant patients undergoing DGF or AGD may become a useful tool for an accurate noninvasive diagnosis of AR.

**Keywords:** Kidney transplantation, Delayed graft function, Acute graft dysfunction, Acute rejection, Tim-3.

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The practice of kidney transplantation has presented significant progress over the last decade. Patient and graft survival had achieved excellent short-term rates, and the use of more efficient immunosuppressive drugs has significantly decreased the incidence of acute rejection (AR). However, episodes of acute graft dysfunction (AGD) are still common and can be due to a variety of causes including a considerable incidence of AR that is associated with lower graft survival (*1*). Also, AR is even more frequent in patients with delayed graft function (DGF) leading to additional detrimental effects on patient and graft survival (*2–6*). The elucidation of graft dysfunction episodes many times requires a biopsy. Furthermore, during the course of DGF, in the absence of a functional parameter, the diagnosis of AR is based on the histologic analysis of graft samples obtained by surveillance biopsies. Graft biopsy, although considered a routine procedure, is uncomfortable and expensive, with a low incidence of complications that can, however, be significant. Also, it is subjected to sampling error, mainly due to the focal nature of the rejection process (*7, 8*).

Transcriptional profiling initially applied to graft tissue, latter provided the opportunity to the development of noninvasive diagnostic tools by analyzing in the peripheral blood and in urinary cells (UC) mRNA from genes associated with the immune response to the graft tissue (*9–16*). A variety of genes evaluated as noninvasive diagnostic markers of AR also led to a better understanding of the clinical and sub clinical AR process (*17, 18*).

T cell immunoglobulin domain, mucin domain (Tim-3), a type 1 membrane protein with a extracellular domain consisting of a immunoglobulin variable region-like domain and a mucin-like region was recently described as molecule selectively expressed on the surface of terminally differentiated T-helper (Th)1 cells and that seems to act in the transport or effector functions of these cells or both (*19*).

In this study, we tested the hypothesis by measuring Tim-3 mRNA in urinary or peripheral blood cells or both, it is possible to diagnose AR in kidney grafts with AGD episodes as well as in grafts with DGF.

## PATIENTS AND METHODS

### Patients and Sample Collection

Kidney transplant recipients with DGF episodes or with AGD who underwent surveillance or per cause graft biopsies were included in this study. Before the biopsy procedure, vascular, urological, nephrotoxic, or infectious causes of graft

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dysfunction were ruled out by Doppler ultrasound, nuclear scans, immunosuppressive drug levels, and urine and blood cultures. Acute dysfunction episodes were suspected in the presence of a significantly confirmed increment of the serum creatinine level. Patients with DGF, defined by the need for dialysis during the first week after transplantation, were submitted to surveillance biopsies every 7 to 10 days until kidney function was recovered or graft was lost. Immediately before each biopsy, peripheral blood and sterile urine samples were collected from patients with diuresis. The kidney biopsies were performed through ultrasound guidance, using a semiautomatic gun with 16-G needle. One and a half fragments were used for histologic analyses and half fragment was immediately frozen in liquid nitrogen and maintained at −70°C. Right after the collection, the blood and UC were isolated and frozen until the RNA extraction. Slide evaluation was performed by a renal pathologist unaware of the clinical data. The Banff '97 classification was used for the histopathologic diagnoses (*20*).

Seventy-nine samples were obtained from 50 patients with DGF who were subsequently classified, according to the histologic findings, as having pure acute tubular necrosis (ATN; n=38) or ATN with superimposed AR (ATN-AR; n=41). Sixty-six samples from 50 patients with AGD, who based on the biopsy results, were subsequently classified as having AR (n=24), calcineurin inhibitor nephrotoxicity (CIN; n=13) or interstitial fibrosis, and tubular atrophy (IFTA; n=29). As a control for gene expression, we also examined mRNA expression in graft tissue, peripheral blood monocyte cells, and UC from 15 kidney transplant recipients with stable graft function who had protocol biopsies, interpreted as normal (NOR), within the first year posttransplantation (NOR; n=15).

All patients received corticosteroids combined with cyclosporine or tacrolimus and mycophenolate (mofetil or sodium) as immunosuppressive therapy. Interleukin-2 antireceptor antibodies (Basiliximab) were given within 24 hr of the transplantation to most patients with DGF. Antilymphocytic antibodies (Thymoglobulin) were given preoperatively to all patients considered as having elevated immunologic risk.

The study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre, accredited by the National Research Council of Brazilian Department of Health, and registered at the Office for Human Research Protections-OHRP-USDHHS (Institutional Review Board-IRB 00000921). Patients gave written informed consent to the procedures and research.

### RNA Extraction

Graft fragments were defrosted, macerated, and processed for the RNA extraction, using QIAamp RNA Blood mini kit (QIAGEN Inc., Chatsworth, CA) method, according to the manufacturer's instructions. Peripheral blood was collected in tubes containing EDTA and the cells were separated with an erythrocyte lysis buffer. UC were obtained by centrifugation at 500*g* for 20 min. The floating material was discarded and cell pellets were resuspended in phosphate-buffered saline solution; centrifuged again for 10 min and stored at −70°C. RNA was extracted from cell pellets isolated from peripheral blood and urine sediment cells by the same method as for the renal tissue. RNA quality was assessed by evaluating the optical density 260-to-280 ratio, and only RNA samples with optical density ratio higher than 1.7 were analyzed, provided that a sufficient amount was available.

### Messenger RNA Quantification

The expression of each gene was analyzed, using the relative quantification technique, by real-time polymerase chain reaction (RT-PCR) using the ABI Prism 7000 (Applied Biosystems, Foster City, CA) detection system. Gene expression assay consists of a mixture of primers and TaqMan MGB (minor groove binding) probes at 360 $\mu$M 20× concentrated. Primers were designed based on the identification of target sequences at the gene bank. The sequences used had already been designed, tested, and validated previously by the manufacturer (Applied Biosystems. Gene expression assays/custom primers and probes). Fluorescent dies used as markers of the probes were 6-carboxy fluorescein (FAM) as reporter (at 5′) and 6- carboxytetramethyl rodamine (TAMRA) as quencher (at 3′). Tim-3 gene (ID: Hs 00262170_m1; gene bank reference 84868) was analyzed along with endogenous molecular controls, used for sample normalization of the mRNA amounts in each reaction. B-actin (PN. 4310881E) was used as an endogenous molecular control, used for sample normalization, and for obtaining an equivalent mRNA amount in each reaction (TaqMan PDAR Endogenous Control).

The reactions were made in duplicates using the TaqMan EZ RT-PCR (PN. N808-0235) (Applied Biosystems) kit, according to the following protocol: 5.0 $\mu$L of 5× TaqMan EZ buffer, 3.0 $\mu$L of manganese acetate (25 mM), 0.75 $\mu$L of dATP (10 mM), 0.75 $\mu$L of dCTP (10 mM), 0.75 $\mu$L of dGTP (10 mM), 0.75 $\mu$L of dUTP (20 mM), 1.0 $\mu$L of rTth DNA polymerase (2.5 U/$\mu$L), 0.25 of AmpErase UNG (1 U/$\mu$L), and pure water to reach a volume of 23 $\mu$L. To this mixture, 1 $\mu$L of primers and probes (20×) and 1 $\mu$L of RNA were added to each reaction, to a final volume of 25 $\mu$L. The cycling program consisted of heating at 50°C for 2 min, 60°C for 30 min followed by heating to 95°C for 5 min and 40 cycles using the temperatures of 94°C for 20 sec and 62°C for 60 sec.

The analyses of amplified products were performed by the relative quantification method $2^{-\Delta\Delta CT}$, which describes alterations to the target gene expression relative to a reference sample (*21*).

### Statistical Analyses

Data are presented as absolute numbers, mean±standard deviations or percentages. Gene quantifications are shown as box-plot representations. Continuous variables with normal distribution were evaluated using analyses of variances followed by the Dunnett's test. The mRNA levels were analyzed using Kruskal-Wallis test with all diagnostic groups. Tukey's test was used for multiple comparisons among the various groups. Mann-Whitney's test was used for comparisons between two groups. Fisher's exact test was used for the analyses of categorical variables. The correlations between the mRNA levels of expression in the different compartments were calculated using the Spearman's correlation test. Receiver operating characteristic curves were generated to find the best cutoff points for the diagnoses of AR (*22*). All analyses were performed using the SPSS (Statistical Package for the Social

Sciences) program (version 14.0, Chicago, IL). The statistical significance level was established as $P$ less than 0.05.

## RESULTS

Table 1 shows the main demographic data of the groups, the number of biopsy samples per group within each histopathologic diagnosis, and relevant transplant information. Seventy percent of the patients received grafts from deceased donors, 60% were male recipients, and 7% received second grafts. Statistical analysis was performed within the major groups. No differences were found in the demographics of the group of patients with DGF. In the group of patients with AGD, differences were found in the time interval to obtain a graft biopsy and serum creatinine, both significantly lower in the group of patients with stable graft function ($P<0.05$), details are shown in Table 1.

In the group of patients with DGF 79 surveillance biopsies were obtained. Forty-one had histopathologic features of ATN with superimposed AR and 38 had features of pure ATN. Serum creatinine levels did not differentiate ATN-AR from ATN (5.6±2.8 vs. 5.9±1.8 mg/dL, respectively; $P$=NS). Both in the graft, peripheral blood and urinary sediment cells the expression levels of Tim-3 mRNA were significantly higher in the group of patients with ATN-AR, as shown in the box-plot graphics in Figure 1 (A–C). The differences of expression reached a significant statistical difference ($P<0.001$). In this group, the diagnostic parameters to the diagnosis of AR were 100% accurate (Table 2). No significant differences in the levels of expression were found in patients with different antibody induction therapy regimens, ATG or Basiliximab, or between them and patients without induction.

In the group of patients with AGD 66 biopsies were obtained. Twenty-four had histopathologic features of AR, 13 of CIN, and 29 of IFTA. Fifteen protocol biopsies interpreted as NOR were used as comparators. Serum creatinine levels were not statistically different between the groups of patients with dysfunction but were significantly lower in the group of patients with NOR biopsies ($P<0.05$). In the graft tissue, peripheral blood and UC, compared with the CIN, IFTA, and NOR groups, Tim-3 mRNA expression was much higher in the group of patients with AR and the differences among the means reached highly significant statistical differences, as shown in Figure 1 (D–F) ($P<0.001$). The diagnostic parameters to the diagnosis of AR are shown in Table 2.

Table 3 shows Spearman's correlation coefficients between Tim-3 mRNA quantifications in the different compartments. Strong and significant correlations were observed between the quantifications in tissue, and blood and urine cells.

In seven patients who had a histopathologic diagnosis of AR and were treated for rejection, a second set of samples was collected. After treatment Tim-3 levels of expression decreased significantly in the graft tissue ($P$=0.001), in the peripheral blood ($P$=0.003), and in the UC ($P$=0.029).

## DISCUSSION

Tim-3 molecule ligates to galectin-9 and this pathway seems to be involved in the effective termination of effector TH1 cells (*23*). In experimental transplantation, the Tim-3 pathway has been suggested to be crucial to the development of peripheral tolerance because its blockage prevents the acquisition of tolerance possibly by dampening the immunosuppressive action of CD4(+)CD25(+) regulatory T-cell populations (*24*, *25*). In the clinical setting, Tim-3 mRNA was demonstrated in increased amounts in renal biopsies and in UC of rejecting grafts and was considered a good marker for clinical AR episodes in renal transplant recipients (*26*, *27*). These characteristics make Tim-3, a good candidate for a

**TABLE 1.** Demographic data of studied groups

| | Groups (no. patients) | | | | | |
|---|---|---|---|---|---|---|
| | DGF (n=50) | | AGD (n=65) | | | |
| | ATN-AR (28/41) | ATN (22/38) | AR (18/24) | CIN (10/13) | IFTA (22/29) | NOR (15/15) |
| Age (yr; mean±SD) | 41±13 | 47±14 | 36±13 | 39±12 | 45±11 | 46±8 |
| Gender (male/female) | 13/15 | 16/06 | 11/07 | 03/07 | 14/08 | 12/03 |
| Transplant number (1st, 2nd) | 26/2 | 21/01 | 16/02 | 10/0 | 19/03 | 15/0 |
| Donor (living/deceased) | 07/21 | 04/18 | 09/09 | 03/07 | 07/15 | 04/11 |
| Cold ischemia time (hr; mean±SD) | 16.7±4.8 | 18.8±5.6 | 21.8±4.3 | 15.2±5.3 | 18.4±4.3 | 15.8±7.7 |
| Time to biopsy (days; mean±SD) | 16±12 | 22±13 | 116±220[a] | 149±169[b] | 664±1148[c] | 101±82 |
| Panel reactive antibodies (%; mean±SD) | 7.9±25.9 | 2.5±11.7 | 0.4±1.8 | 0 | 9±21.5 | 0 |
| HLA mismatches (A, B, DR; mean±SD) | 3.0±1.4 | 3.3±1.3 | 3.1±1.0 | 3.6±1.4 | 2.8±2.0 | 2.6±1.3 |
| Serum creatinine (mg/dL; mean±SD) | 5.6±2.8 | 5.9±1.8 | 3.3±2.2 | 3.6±1.6 | 3.8±1.8 | 1.6±0.4[d] |
| Induction (basiliximab/thymoglobulin) | 18/04 | 16/0 | 6/0 | 6/0 | 08/04 | 10/0 |

None of the other comparisons presented statistically significant differences.
Histopathologic diagnoses ATN-AR, ATN, AR, CIN, IFTA, and NOR are represented as number of patients per samples.
[a] $P<0.05$ vs. IFTA and NOR.
[b] $P<0.05$ vs. IFTA.
[c] $P<0.05$ vs. AR, CIN and NOR.
[d] $P<0.05$ vs. AR, CIN and IFTA.
ANOVA, analysis of variance; ATN-AR, acute tubular necrosis with superimposed acute rejection; ATN, acute tubular necrosis; AR, acute rejection; CIN, calcineurin inhibitor-induced nephrotoxicity; IFTA, interstitial fibrosis and tubular atrophy; NOR, normal; DGF, delayed graft function; AGD, acute graft dysfunction; HLA, human leukocyte antigen; SD, standard deviation.

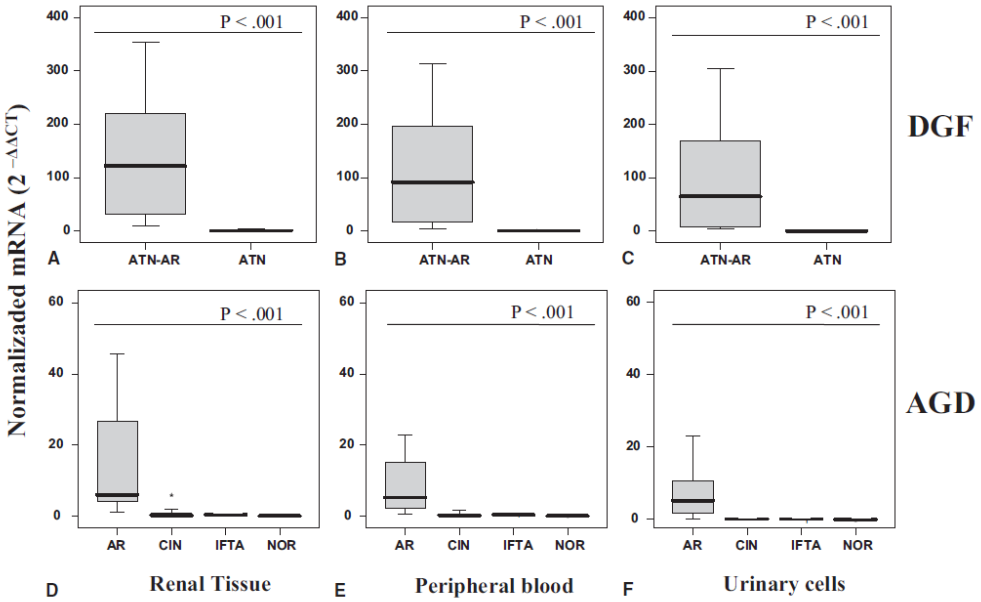


**FIGURE 1.** Tim-3 mRNA expression levels in kidney tissue, peripheral blood leukocytes, and urinary cells in patients with DGF and AGD. The box-plot representation graphics show the medians and the percentile values 10°, 25°, 75°, and 90° for the quantification levels of normalized mRNA ($2^{-\Delta\Delta CT}$). β-actin quantification was used for normalizing the mRNA amounts present in kidney tissue and peripheral blood leukocytes. (A–C) show expression levels in patients with delayed graft function (DGF); (D–F) show expression levels in patients with acute graft dysfunction (AGD); Tim-3 expression levels in both DGF and AGD, in all compartments, were significantly higher in patients with ATN-AR, as compared with patients with ATN (A–C) and also significantly higher in patients with AR compared with patients with CIN, IFTA, and NOR in kidney tissue, peripheral blood, and urinary cells. *P* values are the lowest of the comparisons between each compartment or gene. ATN-AR, acute tubular necrosis with superimposed acute rejection; ATN, acute tubular necrosis; AR, acute rejection; CIN, calcineurin inhibitor-induced nephrotoxicity; IFTA, interstitial fibrosis and tubular atrophy; and NOR, normal.

**TABLE 2.** Areas under the curve, cut off points, and acute rejection diagnostic parameters derived from Tim-3 mRNA quantifications in the peripheral blood and urinary cells of patients with delayed graft function and acute graft dysfunction

| Compartment | AUC (CI 95%) | Cut off | Se | Sp | PPV | NPV | A |
|---|---|---|---|---|---|---|---|
| DGF-urine | 1.00 (1.0–1.0) | 2.5 | 100 | 100 | 100 | 100 | 100 |
| DGF-blood | 1.00 (1.0–1.0) | 3.0 | 100 | 100 | 100 | 100 | 100 |
| AGD-urine | 0.96 (0.91–1.00) | 1.2 | 84 | 96 | 89 | 94 | 93 |
| AGD-blood | 0.96 (0.92–0.99) | 1.8 | 87 | 95 | 87 | 93 | 91 |

Diagnostic parameters are shown in percentages.

DGF, delayed graft function; AGD, acute graft dysfunction; AUC, area under the curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; A, accuracy.

marker of immune mediated allograft injury in the clinical setting and worth testing as a molecular noninvasive test in the peripheral blood mononuclear cells and urinary sediment cells.

Graft dysfunction episodes are frequent in renal transplantation. In this study, we divided it into two settings. First, we evaluated kidney grafts with DGF, where we found that the quantitative analysis of Tim-3 mRNA was extremely accurate in differentiating pure ATN from ATN with superimposed AR. Transcriptional analyses of grafts undergoing DFG has not been described in the literature except for a few reports in a restricted number of patients. By using the competitive PCR method, Li et al., observed that, in 9 of 11 patients with DGF, the perforin and granzyme B expression levels were significantly higher in patients with AR (*15*). Yannaraki et al. (*28*) using the RT-PCR technique, evaluated five patients with

**TABLE 3.** Spearman's correlation coefficients of Tim-3 mRNA quantifications at the different compartments in patients with DGF and AGD

| | Compartments | | | | | |
|---|---|---|---|---|---|---|
| | Peripheral blood-tissue | | Urinary cells-tissue | | Urinary cells-peripheral blood | |
| TIM-3 mRNA | Correlation coefficient | *P* | Correlation coefficient | *P* | Correlation coefficient | *P* |
| DGF | 0.978 | <0.001 | 0.964 | <0.001 | 0.988 | <0.001 |
| AGD | 0.924 | <0.001 | 0.942 | <0.001 | 0.927 | <0.001 |
| All | 0.961 | <0.001 | 0.967 | <0.001 | 0.963 | <0.001 |

Tim-3, T cell immunoglobulin domain, mucin domain; mRNA, messenger ribonucleic acid; DGF, delayed graft function; AGD, acute graft dysfunction.

DGF and reported that the detected amounts of perforin, granzyme B, and fas-ligand were also increased in patients with AR. Later, Renesto et al., analyzing the Tim-3 mRNA expression, pooled patients with DGF in a group along with another causes of dysfunction and also found higher expression in patients with AR (*27*). Finally, Aquino-Dias et al. recently reported that quantitative mRNA analyses of different genes, highlighting FOXP3, could be a useful diagnostic tool in this clinical situation in which a noninvasive method of adequate accuracy would be a cost-effective aid to clinical management (*29*).

Except for the latter work, gene expression analyses in patients with DGF have only been made in UC, which may represent a limitation because many patients might not produce urine for analyses or the urine might come from the native kidneys. In our sample, we were not able to collect urine for analysis for approximately 25% of the occasions. Despite, it was found that when urine is available for mRNA quantitative analyses accurate diagnostic parameters are achievable. However, the possible lack of urine reinforces the need for analyzing gene expression in the peripheral blood, which in this study produced diagnostic parameters identical to the urine analyses.

The authors also analyzed Tim-3 mRNA expression in episodes of graft dysfunction that included AR, CIN, and IFTA. We also found that Tim-3 mRNA expression was an accurate method for the diagnosis of the immune-mediated graft injury present during AR episodes. In the peripheral blood cells and in the UC, the expression levels were much higher during AR and correlated with values obtained from graft tissue analyses.

The evaluation of mRNA amounts from peripheral blood leukocytes of kidney transplants patients with AGD was initially performed by Vasconcellos et al. (*12*) in studies in which mRNA was quantified by reverse transcription competitive PCR, who demonstrated that these evaluations could produce proper parameters to the diagnosis of AR.

The mRNA analyses of urine-sediment cells of kidney transplant patients are interesting because lymphocytes present in urine, under most circumstances, have trafficked through the kidney, so the status of these cells might accurately reflect the status of graft-infiltrating lymphocytes. Gene profiling in the urine have been performed by Muthukumar et al. who evaluated FOXP3 and perforin mRNA in the urine of kidney transplant recipients and found that the levels of these genes were significantly higher in patients with AR, as compared with groups with histologic diagnoses of chronic graft nephropathy and transplants with stable function (*16*). Also, Renesto et al. (*27*) have demonstrated increased amounts of Tim-3 mRNA in UC of rejecting grafts and considered the quantification of this gene, a good marker of AR in renal transplant recipients.

We acknowledge that in addition to AR other conditions, mainly acute CIN and pyelonephritis, might occur during DGF and play an important role in delaying functional graft recovery. However, at least regarding to CIN, the data obtained in patients with AGD suggest that increased expression would not occur.

The findings of this study allowed the conclusion that in patients with DGF or AGD quantification of Tim-3 mRNA, in peripheral blood leukocytes or in urine-sediment cells provides an accurate marker for the presence of AR. As previously suggested the validation of these results in multicenter longitudinal study and possible sophistications of used techniques might allow transcriptional profiling to replace or reduce the need for kidney biopsies for evaluation of graft dysfunction and during DGF, and evaluate its applicability to anticipate the diagnosis of AR (*30*).

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## 8.2. TCLE do projeto 07-548

ANEXO 2.

**Termo de Consentimento Livre e Esclarecido**

A unidade de Transplante Renal do Hospital de Clínicas de Porto Alegre pelo presente termo de consentimento **convida** o (a) Sr (a) _____ a participar de um estudo que será elaborado com o **objetivo** de desenvolver melhores métodos para o diagnóstico da rejeição de transplantes renais e de forma mais rápida. Os procedimentos a que os (as) Sr (as) serão submetidos são coleta de biópsias renais (por necessidade clínica). Junto com a biópsia serão coletados sangue e urina.Estes materiais serão submetidos à análises moleculares no laboratório do serviço de Nefrologia. Informamos que os riscos envolvidos na pesquisa são os mesmos envolvidos no transplante renal e o risco mínimo representado pelas biópsias renais. Pode ocorrer em até 2% dos casos, sangue na urina depois da coleta, que não necessitam tratamento específico. Risco de hemorragia grave é raro ocorrendo em menos de 1% dos pacientes submetidos à biópsias. As coletas de sangue (por punção da veia do braço) e urina não oferecem risco, podendo ocorrer apenas um pequeno hematoma na área da punção da veia. Nenhum benefício financeiro será obtido na participação do presente estudo tanto para a unidade de transplante renal, quanto para os (as) Sr (as), mas sua ajuda será importante na pesquisa de tratamentos melhores para a rejeição de futuros pacientes. Para seu esclarecimento a qualquer momento ou em caso de necessidade o Sr (a) poderá entrar em contato com os membros da equipe da unidade de transplante renal pelos telefones 2101-8295, 2101-8121 ou ainda pelo telefone celular do Dr. Roberto Ceratti Manfro - 99752461. Ainda em caso de urgências com o plantão da Nefrologia Transplante do HCPA telefone 21018000.

Eu, _____ aceito a participar do estudo _____

e declaro que fui também informado:

- Da garantia de receber resposta a qualquer pergunta sobre o presente estudo;

HCPA / GPPG
VERSÃO APROVADA
11/12/07
07548

GPPG - Recebido
07 DEZ 2007
Por Janice nº 07548

- Da liberdade de retirar meu consentimento, a qualquer momento, sem que isto traga prejuízo à continuação de meu cuidado e tratamento;
- Do caráter confidencial das informações relacionadas com minha privacidade;
- Da disponibilidade de tratamento médico caso existam complicações causadas por esta pesquisa;
- De que não terei despesas por participar do estudo.

| | |
|---|---|
| Assinatura do paciente | Assinatura do pesquisador<br>Roberto C. Manfro<br>(51) 2101-8295 |

## 8.3. Ficha de coleta das amostras dos pacientes

**Protocolo de Coletas do Estudo de Modulação da Expressão Gênica em transplantados renais**

**Ficha de Coleta do Paciente** Ficha:_____

Nome:_____ Tx _____/_____

Data Tx:____/____/____ Prontuário:_____ Doador: ( ) Vivo _____( ) Cadáver

Data Nascimento: ____/___/___ Idade: _____ TCLE:_____

HLA doad→ **A:**_____ **B:**_____ **DR:**_____
HLA recept→ **A:**_____ **B:**_____ **DR:**_____

Painel: → Pico: IF: Anast: Doença de Base:
↘ Último: IMS inicial: Tx prév: Transf:

Gestações:

**Data das Coletas:**

**S U**
**( ) ( ) 0 →**
**( ) ( ) 3 →**
**( ) ( ) 5 →**
**( ) ( ) 10→**
**( ) ( ) 15→**
**( ) ( ) 20→**
**( ) ( ) 25→**
**( ) ( ) 30→**
**( ) ( ) 45→**
**( ) ( ) 60→**
**( ) ( ) 90→**

**DGF:( )sim ( )não Bx: ( )sim ( )não**

**Outras:** **n° de amostras:_____**

| **Dia Pós Tx:** | **Amostras Coletadas:** |
|---|---|
| _____ | ( ) Tecido Renal ( ) Sangue EDTA 5mL (tampa roxa) ( ) urina (>30mL) |
| _____ | ( ) Tecido Renal ( ) Sangue EDTA 5mL (tampa roxa) ( ) urina (>30mL) |
| _____ | ( ) Tecido Renal ( ) Sangue EDTA 5mL (tampa roxa) ( ) urina (>30mL) |
| _____ | ( ) Tecido Renal ( ) Sangue EDTA 5mL (tampa roxa) ( ) urina (>30mL) |
| _____ | ( ) Tecido Renal ( ) Sangue EDTA 5mL (tampa roxa) ( ) urina (>30mL) |

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