UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

Caracterização de novas ferramentas diagnósticas em imunologia de transplantes para avaliação de candidatos à transplante renal.

JAMILE ABUD

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Orientador: Prof. Dr. Roberto Ceratti Manfro

Tese de doutorado apresentada como requisito parcial para obtenção de título de Doutor em Medicina: Ciências Médicas, da Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Medicina: Ciências Médicas.

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Epígrafe:

"A característica singular do indivíduo nos dissuade inteiramente de tentar o transplante entre duas pessoas distintas. Tal é a força e o poder da individualidade que se alguém disser que acredita que pode conseguir o mínimo sucesso em uma operação destas, ou é um grande supersticioso, ou trata-se de alguém com muito mau embasamento científico."

Gaspare Tagliacozzi

De Curtorum Chirurgia per Insitionem – 1596

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RESUMO

Introdução: Atualmente, a região sul é a segunda região do Brasil em número de transplantes renais (1276 transplantes em 2017). Diversos tipos de complicações podem encurtar a sobrevida de pacientes e enxertos, entre essas situam-se as rejeições celulares e as mediadas por anticorpos. As provas cruzadas identificam anticorpos que podem ocasionar as rejeições, são elas: Citotoxicidade Dependente de Complemento (CDCXM) e prova cruzada por Citometria de Fluxo (CFXM). O advento do teste de fase sólida *Single Antigen Beads*, permitiu a realização da prova cruzada virtual (VXM) a partir de 2003.

Objetivos: O objetivo geral do estudo foi avaliar clínica e laboratorialmente os novos protocolos para a avaliação imunológica pré-transplante renal. Os objetivos específicos foram avaliar a correlação laboratorial entre os métodos (CDCXM, CFXM e VXM) e realizar a validação clínica da utilização do teste CFCXM como teste único no pré-transplante.

Metodologia: Um estudo diagnóstico retrospectivo foi realizado para a correlação laboratorial das provas cruzadas. Foram coletados os resultados das provas cruzadas do Laboratório de Imunologia de Transplantes da Santa Casa de Porto Alegre do ano de 2010. A validação clínica foi realizada estabelecendo duas coortes: uma coorte prospectiva de pacientes transplantados renais selecionados com CFXM (teste) e uma coorte retrospectiva de pacientes transplantados renais selecionados com o CDCXM (controle). Foram avaliados desfechos laboratoriais clínicos coletados nos laboratórios da Santa Casa de Porto Alegre e nos prontuários eletrônicos dos pacientes.

Resultados: Em 78 doadores falecidos foram analisadas 713 provas cruzadas no ano de 2010 contemplando os três métodos em uma média de 6,74 ± 3,38 pacientes por doador. Dos 486 (68,2%) testes CDCXM negativos, 62 (12,8%) foram positivos na CFXM e destes, 13(1,82%) não tinham anticorpo anti-HLA doador-específico conhecido. Na avaliação dos desfechos clínicos, 97 pacientes transplantados renais com CFXM e 98 pacientes transplantados renais na coorte controle foram acompanhados por 1 ano. Todos os transplantes foram realizados com órgãos de doadores falecidos e todas as provas cruzadas na coorte controle foram negativas. Um paciente da coorte CFXM foi positivo para linfócitos B. Em

um ano de acompanhamento, não houve diferença significativa na sobrevida dos pacientes (*p*=0,591) e na sobrevida dos enxertos (*p*=0,692) entre os grupos.

Conclusões: A correlação entre CDCXM e CFXM foi satisfatória e pela semelhança de sensibilidade, concluímos ser adequada a utilização da CFXM e VXM na avaliação pré-transplante. Além disso a frequência de resultados falsos positivos na CFXM foi considerada baixa. Quando comparamos a sobrevida dos pacientes e dos enxertos entre as coortes controle e CFXM não encontramos diferença significativa. A partir destes achados concluímos que a utilização da CFXM como teste único não modificou os desfechos clínicos em um ano de acompanhamento, sugerindo segurança na sua utilização em conjunto com a VXM.

Termos-Chave: Transplante Renal, Rejeição Mediada por Anticorpos, Citotoxicidade Dependente de complemento, Citometria de Fluxo, Prova Cruzada Virtual.

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ABSTRACT

Background: Currently Brazil's southern region is the second in number of kidney transplants in the country (1276 transplants in 2017). However, several types of complications can shorten the survival of patients and grafts, among which are the antibody-mediated rejections. Crossmatching tests identify antibodies that can cause rejection, such as Complement Dependent Cytotoxicity (CDCXM) and flow cytometric crossmatch (FCXM) test. Single Antigen Beads solid phase assay allowed the virtual crossmatch (VXM) to be performed starting in 2003.

Aim: The main purpose of the study was evaluate and validate the new crossmatching protocols for kidney pre-transplantation. The first specific aim was to evaluate laboratory correlation between the methods (CDCXM, FCXM and VXM). We analyzed accuracy and the percentage of false-positive tests. The clinical validation of the use of the FCXM test as a single test in the pre-transplantation (second specific aim) was performed in patients that underwent kidney transplantation.

Methods: A retrospective diagnostic study was performed for the laboratory correlation of the crossmatches tests; we collected the results of these tests in the Transplant Immunology Laboratory, at Santa Casa of Porto Alegre of the year 2010. The clinical validation was performed by establishing two cohorts: a prospective study cohort of kidney transplant patients evaluated by FCXM and a retrospective cohort of kidney transplant patients evaluated by CDCXM (control). Clinical and laboratory outcomes were evaluated. Data were collected in medical charts in the Santa Casa de Porto Alegre.

Results: In 78 deceased donors, 713 crossmatches were analyzed in the year 2010, considering the three methods in an average of 6.74 ± 3.38 patients per donor. Of the 486 (68.2%) CDCXM negative tests, 62 (12.8%) were positive in FCXM and of these, 13 (1.82%) had no known anti-HLA donor antibody. In the test cohort, 97 patients transplanted with FCXM and 98 patients kidney transplants in the control cohort, were followed for 1 year. All transplants were performed with deceased donors organs and all crossmatches in the control cohort were negative. One patient in the cohort FCXM was positive for B

lymphocytes. At one year follow-up, there was no significant difference in patient survival ($p = 0.591$) and graft survival ($p = 0.692$) between groups.

Conclusions: The correlation between CDCXM and FCXM was satisfactory and due to the similarly of sensitivity, we concluded that the use of FCXM and VXM in the pre-transplantation evaluation was adequate. When we compared the survival of patients and grafts between the CDCXM and FCXM cohorts, we did not find a significant difference. Therefore, the use of FCXM as a single test did not modify the clinical outcomes at one year of follow-up, suggesting that this test, along with the VXM, ensures safe kidney transplantation.

Keywords: Kidney Transplant, Antibody-Mediated Rejection, Complement-Dependent Cytotoxicity, Flow Cytometric, Virtual Crossmatch.

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AAD	Anticorpo Anti-Doador
AGH	Antiglobulina Humana
C4d	Fração 4d do complemento
CDCXM	Prova Cruzada por Citotoxicidade Dependente de Complemento
CFXM	Prova Cruzada por Citometria de Fluxo
DSA	*Donor Specific Antibody*
DTT	Ditiotreitol
FCXM	*Flow Cytometry Crossmatch*
HLA	Human Leukocyte Antigen
IgM	Imunoglobulina M
IgG	Imunoglobulina G
MHC	Major Histocompatibility Complex
RHA	Rejeição Humoral Aguda
SAB	*Single Antigen Beads*
VXM	Prova Cruzada Virtual

SUMÁRIO

1. INTRODUÇÃO

O transplante de órgãos é uma terapêutica que objetiva a substituição de órgãos que perderam a sua função no organismo[1].

Atualmente o Brasil é o segundo país no mundo em número absoluto de transplantes renais. Em 2016, realizaram-se 5.426 transplantes renais (26,9 por milhão de pessoas) no nosso país, entretanto o número de pacientes em lista de espera, neste mesmo ano, foi aproximadamente quatro vezes acima. A região Sul é a segunda região do Brasil em número de transplantes renais. Em 2017 foram realizados nessa região 1.276 transplantes com doadores falecidos e 173 transplantes com doadores vivos. O número de pacientes em lista de espera na região Sul, em dezembro de 2017, era de 2.374 pacientes[2].

Em 1969 Patel e Terasaki demonstraram que a presença de anticorpos anti-HLA doador específico (AAD) no soro de receptores no pré-transplante foi o maior fator de risco para rejeição hiperaguda e não função primária do enxerto[3]. Esse achado foi o ponto de partida para a abertura de laboratórios de histocompatibilidade e estabelecimento da prova cruzada de linfócitos T como teste imunológico pré- transplante com subsequente impacto na diminuição de rejeições hiperagudas. Um resultado negativo justificava o procedimento, mas um teste positivo era considerado como contraindicação ao transplante[4]. A prova cruzada por Citoxicidade Dependente de Complemento (CDCXM) foi o teste precursor na identificação de AAD nos anos 1970. Ao logo dos anos a busca por uma maior sensibilidade fez com que o protocolo sofresse o acréscimo da anti-globulina humana (AGH)[5]. O aumento adicional de sensibilidade para a prova cruzada foi obtido com o desenvolvimento da prova cruzada por citometria de fluxo (CFXM)[6]. No início dos anos 80, a citometria de fluxo surgiu como uma importante tecnologia no laboratório clínico, com um aumento de sensibilidade em relação ao CDCXM[6;7].

Em 2003 foi descrito o teste de fase sólida que identifica anticorpos anti-HLA através da metodologia Luminex, propiciando a realização da prova cruzada virtual (VXM), que consiste na possibilidade de inferir o resultado da prova cruzada baseado no rastreamento e identificação de anticorpos HLA [4; 8].

O avanço das metodologias em termos de sensibilidade, CFXM e VXM, altera a interpretação clínica da avaliação imunológica pré-transplante. Por este motivo, se faz importante a validação laboratorial e clínica destes métodos em cada centro transplantador. As abordagens atuais visam a estratificação de risco com base na identificação de anticorpos e resultados de provas cruzadas, levando em consideração o tipo de órgão e considerações clínicas, tais como urgência, estratégias de imunossupressão disponíveis e condições clínicas do doador[6].

2. REVISÃO DA LITERATURA

2.1. Estratégia de busca e seleção da informação

Esta revisão da literatura está focada nos aspectos relacionados a rejeição e perda do enxerto nos transplantes renais sendo a causa de origem imunológica identificada através dos testes imunológicos pré-transplante. A estratégia de busca envolveu a base de dados PubMed no período de 2001 a julho de 2018. Foram realizadas buscas através dos termos "*FlowCrossmatch", "Virtual Crossmatch", "CDC Crossmatch", "KidneyTransplantation" and "Antibody-mediatedrejection"* e suas combinações (Figura 1).

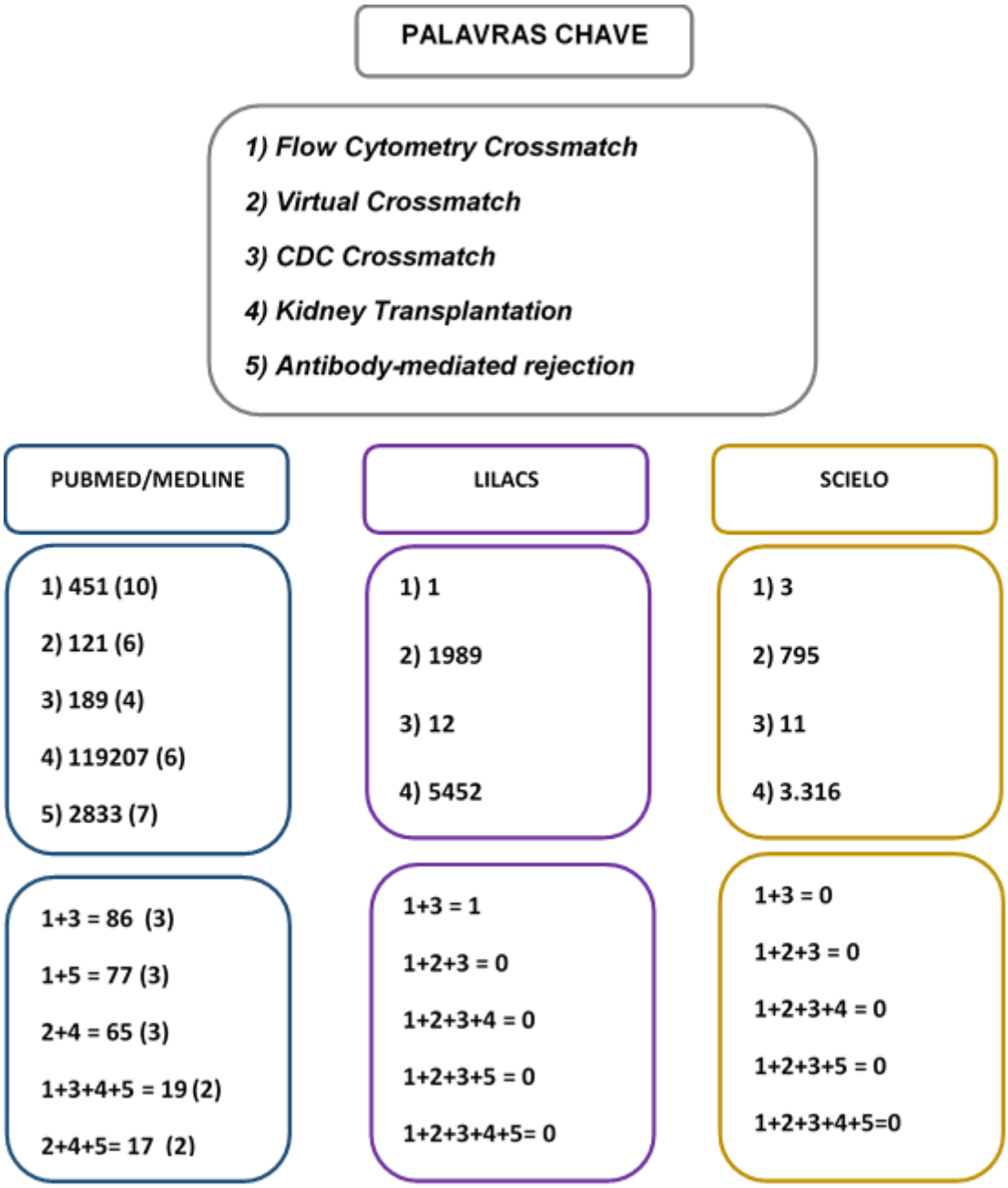


Figura 1: Estratégia de busca para revisão da literatura. Entre parênteses número de referências utilizadas. Elaborado pela autora.

2.2. Transplante: da mitologia à complexidade gênica

De acordo com a mitologia grega, o primeiro transplante teria sido realizado na ilha de Creta. A bíblia sagrada e outros textos cristãos contam com muitos relatos sobre o transplante de partes do corpo humano, no entanto, o relato mais célebre fala dos gêmeos São Cosme e São Damião, que viveram entre os anos 285 e 305 da era Cristã. A lenda conta que os irmãos na tentativa de curar a perna de um cristão que se encontrava necrosada por um "cancro" – termo que poderia significar câncer ou gangrena – usaram a perna de um etíope, recém falecido, para um transplante. O feito é conhecido como o "milagre de São Cosme e Damião" e retratados em dezenas de pinturas da Idade Média (Figura 2). A partir de 1880, o transplante de órgãos, aliado ao correspondente conhecimento a respeito da natureza do corpo e da doença, surge e difere fundamentalmente dos transplantes realizados há séculos na cirurgia plástica, em que o cirurgião substitui partes lesadas da superfície corporal. Com o transplante renal é que os transplantes de órgãos se iniciaram, a partir da segunda metade do século XX [1].

Figura 2: ."A cura de Justiano por São Cosme e Damião"[1]

O primeiro transplante renal com sucesso foi realizado em Boston, em dezembro de 1954, entre gêmeos idênticos, sem nenhuma forma de imunossupressão tendo o receptor sobrevivido por 8 anos. Entretanto, os transplantes renais ingressaram na prática médica apenas a partir de 1960, com a descoberta e o emprego de medicações imunossupressoras. O primeiro transplante renal no Brasil foi realizado em abril de 1964 no Hospital dos

Servidores do Estado, no Rio de Janeiro enquanto que no Hospital das Clínicas de São Paulo, em janeiro de 1965, foi desenvolvido o primeiro programa de transplante renal do país [1].

O transplante renal é uma prática multidisciplinar onde cada área contribui de forma importante para o sucesso do procedimento e manutenção do enxerto. Dentre elas, está a avaliação imunológica pré-transplante, responsável pela predição de risco, e acompanhamento de rejeições humorais no período pós transplante.

O sucesso dos transplantes iniciou na metade do século XX e um fato responsável pelo mesmo foi o conhecimento da imunologia de transplantes através da descrição do sistema HLA (*humanleukocyteantigen*) no início dos anos 1950, com os trabalhos de Snell, Benacerraf e Dausset. Os antígenos presentes nos tecidos geneticamente diferentes e os genes que codificam estes antígenos são conhecidos por antígenos e genes da histocompatibilidade. A região que codifica esses genes é denominada de complexo principal de histocompatibilidade (MHC – *major histocompatibilitycomplex*) e está localizada no braço curto do cromossomo 6. As moléculas codificadas nos genes do MHC são constituídas estruturalmente por glicoproteínas e estão divididas em três grupos[8;9].

O primeiro grupo é chamado MHC classe I, é constituído por uma longa cadeia polipeptídica (43 kD) e sempre acompanhada, não covalentemente, por uma pequena proteína não polimórfica (12kD) denominada beta-2 microglobulina e codificada em humanos, no cromossomo 15. Os genes MHC classe I melhor definidos e de importância conhecida para o transplante de órgãos codificam as proteínas HLA-A, HLA-B, HLA-C. Os antígenos HLA classe I estão presentes na membrana de todas as células nucleadas do organismo, sua função fisiológica é apresentar aos linfócitos T citotóxicos, peptídeos oriundos do metabolismo proteico da célula, podendo ser ou não antígenos. O MHC classe II é o segundo grupo, composto por duas glicoproteínas distintas (34kD e 27kD) chamadas de cadeias alfa e beta. Esse grupo apresenta três principais genes que codificam as proteínas HLA-DRB1, HLA-DQB1 e HLA-DPB1, relevantes aos transplantes. Estas estão presentes em algumas células apresentadoras de antígenos: linfócitos B, monócitos, macrófagos e células epiteliais tímicas. Podem ser expressos, ainda, em células T ativadas e em

endotélio de vasos capilares (Figura 3). O terceiro grupo, MHC classe III é constituído de uma série de proteínas solúveis presentes no soro: algumas proteínas do sistema complemento, fator de necrose tumoral e proteínas de choque térmico, além de algumas proteínas não diretamente relacionadas ao sistema imune [10].

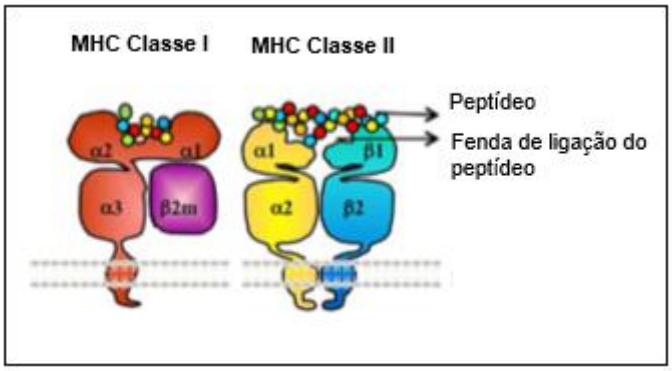


Figura 3: Estrutura molecular HLA Classe I e II. Figura modificada[9].

2.3. A evolução dos métodos de histocompatibilidade

A descoberta do HLA resultou diretamente na teoria humoral da transplantação[8]. A rejeição humoral aguda (RHA) é geralmente causada por aloanticorpos anti-HLA pré-formados e ou anticorpos "*denovo*", é a principal causa de injúria imunológica precoce ou tardia do enxerto[11]. O diagnóstico da RHA é embasado na demonstração morfológica de injúria tecidual, depósitos de C4d em capilares peritubulares e evidências de atividade humoral específica contra o doador: presença de DSA, do inglês *Donor Specific Antibody*[12].

A sensibilização, ou seja, a formação de anticorpos anti-HLA pode ocorrer nas gestações, transfusões de sangue e transplante prévio[1;9].

A teoria humoral foi documentada ao longo da evolução de métodos laboratoriais: viabilidade Celular (1914), microlinfocitotoxicidade (1964) e o teste SAB do inglês *Single Antigen Beads* (2003)[8]. Progressivamente, ao longo da prática dos transplantes, esses testes foram incorporados na rotina de avaliação laboratoriais pré-transplante renal e de outros órgãos. Acompanhados por um melhor entendimento da imunobiologia e genética da aloresposta esses testes permitem uma avaliação mais segura dos riscos imunológicos envolvidos na

transplantação. No entanto, alguns aspectos necessitam melhor entendimento e avaliação, de maneira a simplificar as testagens, mantendo-lhes a segurança.

A avaliação imunológica pré-transplante tem por objetivo evitar as rejeições. A rejeição hiperaguda é evitada com a verificação da compatibilidade entre doador e receptor quanto ao sistema ABO e ao sistema HLA e por meio da realização de prova cruzada[1].

A prova cruzada é conceitualmente uma simples reação entre antígenos do doador e os AAD(s) do receptor quando esses estão presentes no soro do mesmo[1,4]. A metodologia pioneira utilizada para prova cruzada foi o CDCXM, descrito por Terasaki and Patel em 1969[3]. O CDCXM pode ser realizado de linfócitos totais ou após a separação das subpopulações de linfócitos T e B do doador. Consiste de uma primeira incubação do soro do receptor (anticorpos) com os linfócitos do doador (antígenos HLA). Complemento de coelho é adicionado e uma segunda incubação é realizada, ativando a via clássica de complemento, resultando no rompimento dos linfócitos nas reações positivas. A perda da integridade da membrana celular é visualizada através de um microscópio invertido de fluorescência, após a adição de um corante vital. O percentual de células lisadas é reportado em *scores* (0,1,2,4,6,8) conforme consenso no *International Histocompatibility Workshop* (IHW) [13].

O CDCXM é usualmente modificado pelo acréscimo da AGH. A adição de um anticorpo de origem animal, específico contra imunoglobulina humana, amplifica a densidade de anticorpos fixados à superfície da célula alvo (antígeno), tornando possível a ativação do complemento e consequente rompimento da célula em situações que seriam consideradas negativas pela baixa concentração de anticorpos anti-doador, aumentando assim a sensibilidade do teste (Figura 4). Outro importante avanço nesta técnica foi a introdução do ditiotreitol (DTT). Esse agente químico reduz a imunoglobulina IgM, tornando-a incapaz de ativar o complemento, sem interferir na função das IgG (s). Assim, caso a prova continue positiva após a adição de DTT, podemos atribuir a reação a presença de IgG. Anticorpos da classe IgM geralmente não são considerados na sensibilização por não serem deletérios aos aloenxertos. Por outro lado, transplantes renais não devem ser realizados se a CDCXM for positiva para anticorpos anti-HLA da classe IgG[5;14].

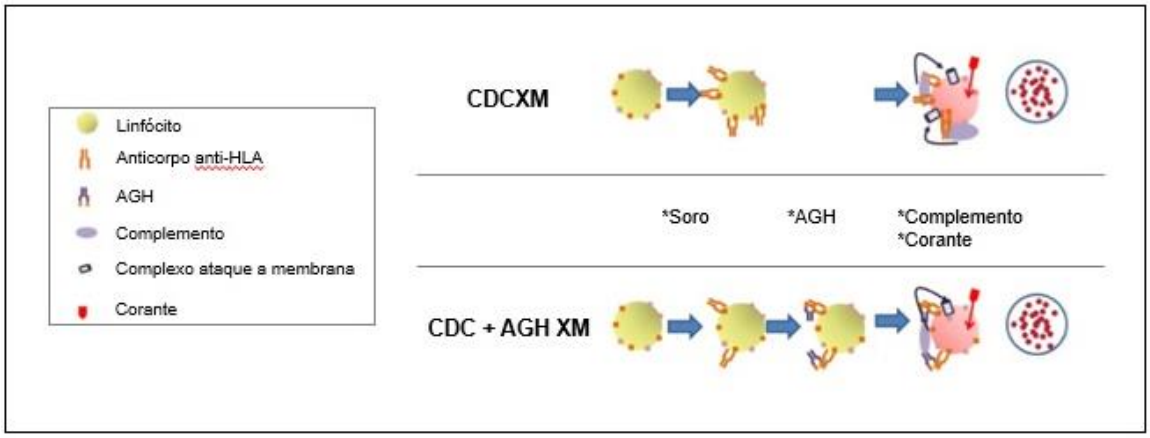


Figura 4: Princípio da técnicaCDCXM e AGH+CDCXM. Figura adaptada[4].

Modificações foram realizadas na CDCXM na tentativa de aumentar sua sensibilidade, dentre elas estão a inclusão de etapas de lavagens (para remoção de interferentes), aumento no tempo de incubação (para permitir a ligação com anticorpos de baixa avidez) e adição de AGH [14]. Entretanto a ocorrência de episódios de rejeição humoral aguda sugere que pacientes que foram sensibilizados e com baixo nível de imunização não são detectáveis pela CDCXM [15].

No início dos anos 80, surgiu a CFXM como uma importante ferramenta no laboratório de histocompatibilidade, propiciando aumento de sensibilidade em relação a CDCXM [7].

A citometria de fluxo é realizada através da incubação dos linfócitos do doador com o soro do receptor. Se anticorpos anti-HLA específicos contra o doador estiverem presentes, eles se depositarão na superfície dos linfócitos. A identificação dos aloanticorpos ligados se dá através da adição de um anticorpo antiimunoglobulina humana marcado com um fluorocromo. As subclasses de linfócitos podem ser diferenciadas utilizando-se anticorpos monoclonais específicos que marcam linfócitos T (CD3) ou linfócitos B (CD19) [16]. As leituras das reações e as análises das amostras são realizadas por um equipamento automatizado, chamado de citômetro de fluxo (Figura 5). Esse equipamento permite analisar grande quantidade de células de maneira individual e com alta sensibilidade analítica[5]. Os resultados são quantitativos e interpretados a partir de um ponto de corte. Uma CFXM positiva representa risco, mas não

necessariamente uma contraindicação ao transplante[6]. A tabela 1 resume a interpretação da CFXM.

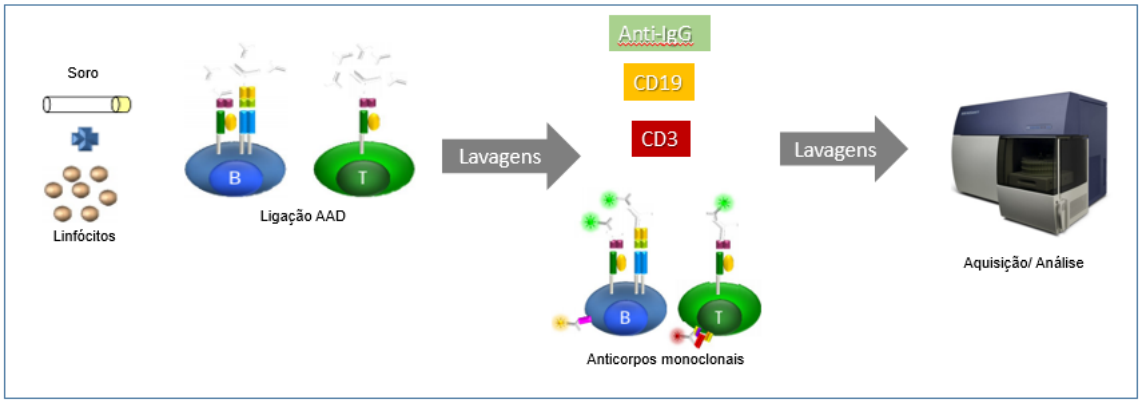


Figura 5: Princípio da técnica CFXM. Elaborada pela autora.

Tabela 1: Padrões de reatividade na CFXM.

Linfócitos T	Linfócitos B	Interpretação[a]
Negativo	Negativo	Ausência de anticorpos anti- HLA ou títulos muito baixos
Negativo	Positivo	Predominantemente anti-HLA classe II
Negativo	Fracamente positivo	Baixos títulos de anti-HLA classe I ou classe II
Positivo	Positivo	Presença de anti-HLA classe I ou a combinação de classe I ou II
Positivo	Negativo	Possivelmente se trata de uma reação não HLA

Legenda:[a] considerando que o resultado foi confirmado com teste de fase sólida. Tabela adaptada[16].

A CFXM é uma técnica de difícil padronização em função da variabilidade entre os citômetros, fluorocromos, reagentes de antiglobulinas e variações na relação célula-soro[6]. Além da sensibilidade, de acordo com a produtividade do laboratório a CFXM otimiza o tempo de execução da prova cruzada quando um número grande de amostras é testado e por permitir que sejam testados linfócitos T e linfócitos B numa única reação [17].

Em 2011 foi proposto um protocolo otimizado a partir do protocolo original de CFXM, chamado protocolo HALIFAX [18;19]. Entre outras modificações, está a redução dos tempos de incubação e centrifugação, o que propicia uma reduçãodotempodeexecuçãodoteste. A importância da redução do tempo de execução da prova cruzada está relacionada com a contribuição da mesma no tempo de isquemia fria, inerente aos transplantes com doadores falecidos e é um dos principais fatores preditores da função inicial dos enxertos renais [20].

Adicionalmente as provas cruzadas tradicionais, utiliza-se a prova cruzada virtual na avaliação do risco imunológico. A VXM consiste na identificação de AAD sem a realização da prova cruzada real. Para tal é necessário que se conheça os antígenos HLA dos doadores para verificar a presença de AAD no soro do receptor. A identificação e semiquantificação de AAD no soro dos receptores é realizada pelo teste de fase sólida com alta sensibilidade, o teste SAB para detecção de anticorpos, utilizados na técnica denominada Luminex®. O teste SAB utiliza microesferas (“*beads”)* de poliestireno revestidas com dois marcadores fluorescentes (classificadores de sinais) resultando, teoricamente, em mais de 100 distintas populações de *beads*. Cada população de microesferas é revestida com moléculas de um único clone alélico de antígenos HLA de classe I ou classe II, permitindo assim uma análise precisa de anticorpos específicos. A antiglobulina no teste com microesferas é marcada com um terceiro marcador fluorescente (tradutor de sinal) (Figura 6). A análise do teste é feita por um equipamento da plataforma Luminex e o nível de anticorpos anti-HLA específicos ligados às microesferas é expresso pela média da intensidade de fluorescência do sinal reportado [6].

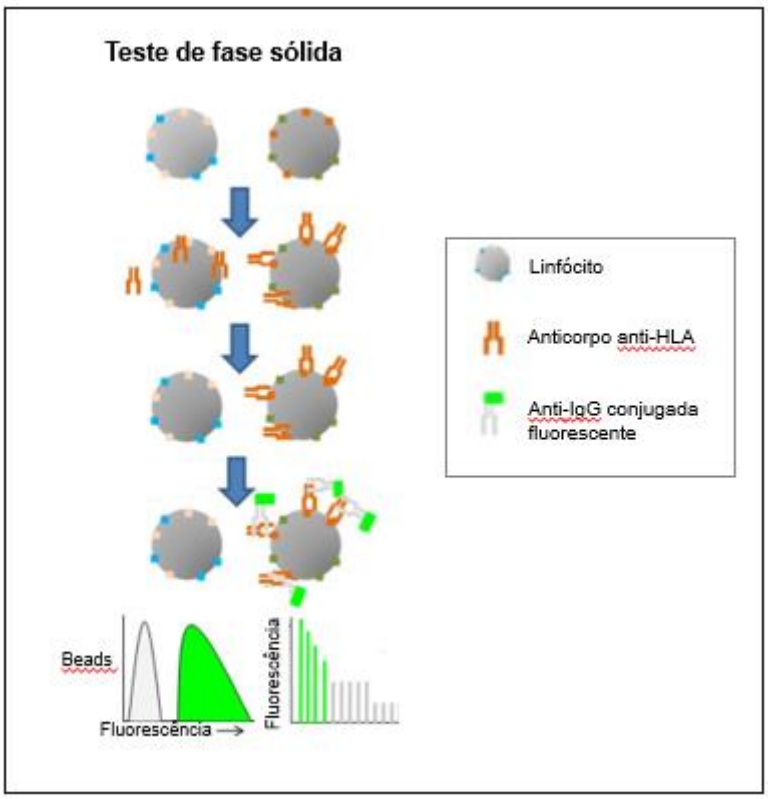


Figura 6: Princípio do teste SAB. Figura adaptada[4].

A implantação da VXM no passado foi problemática em função da baixa sensibilidade das técnicas empregadas e das imprecisões na tipificação HLA. O avanço da tecnologia na identificação de anticorpos anti-HLA usando SAB e a maior segurança que as técnicas de biologia molecular trouxeram às tipificações, permitem hoje uma acurada predição de resultado da prova cruzada [21].

O aumento da sensibilidade fez com que os grupos transplantadores modificassem a conduta clínica frente a um resultado de prova cruzada. Diversos estudos retrospectivos foram realizados na tentativa de entender a acurácia dos métodos isoladamente e concomitantemente.

Karpinski*et al*. (2001) realizaram CFXM e painel de reatividade de anticorpos retrospectivamente em 143 pacientes com CDCXM negativos. Dos pacientes testados, 18 (13%) tiveram CFXM positivos para linfócitos T, destes 6 pacientes tiveram perda precoce do enxerto por rejeição mediada por anticorpos e 12 pacientes as causas foram outros eventos pós transplante. O painel de reatividade de anticorpos apresentou positividade em 8 dos 18 pacientes CFXM positivo. Os autores sugerem que em pacientes submetidos pela primeira vez a

transplante, um CFXM linfócitos T positivo pode representar uma relativa contraindicação ao transplante [22].

Em um estudo de Ho *et al*. (2008) avaliaram comparativamente sensibilidade e especificidade dos testes CDCXM, CFXM, VXM e relevância clínica dos mesmos. Foram incluídos 354 casos consecutivos de receptores de transplante renal com órgãos de doadores falecidos. A comparação da correlação entre perda de enxerto, presença de DSA e resultado de *crossmatch* indicaram que a sensibilidade foi 5%, 16% e 17% enquanto a especificidade foi 99%, 93% e 86% em CDCXM, VXM e CFXM respectivamente. A conclusão foi que o CDCXM é um teste mais confiável e otimiza a chance dos pacientes de receber o enxerto, já o CFXM e o VXM são de grande importância para a identificação de pacientes que necessitam monitoramento por biopsia e sorologia para diagnóstico e tratamento precoce da rejeição mediada por anticorpos [23].

Wu *et al.* (2013) em um estudo retrospectivo avaliaram a relevância clínica da presença de DSA com baixa intensidade de fluorescência no pré- transplante. Entre 221 pacientes, todos com CDCXM negativos, 11(5%) apresentaram rejeição mediada por anticorpos em até 200 dias após o transplante. Foram utilizados múltiplos pontos de corte 500, 1000, 2000, 3000 e 5000 cujas significâncias estatísticas para desfecho foram respectivamente p= 0.003, 0.001, 0.007 e 0.003. Os dados sugerem a identificação de DSA em baixos níveis de fluorescência, antes e após o transplante, pelo teste SAB pode ter um valor preditivo positivo na rejeição mediada por anticorpos e perda do enxerto[24].

O primeiro kit e mais difundido nos laboratórios de histocompatibilidade para identificação de anticorpos anti-HLA foi o SAB desenvolvido pela LABScreen, One Lambda. Um estudo transversal realizado no Hospital de Clínicas de Porto Alegre comparou o kit SAB com o kit Xm-DSA *Tepnel Lifecodes Corporation* em 122 pacientes transplantados renais. Foram detectados AAD HLA classe I em 17 pacientes (13,9%) e AAD HLA classe II em 22 pacientes (19,6%) com o kit SAB, enquanto que no Xm-DSA foram identificados DSA (s) HLA classe I e II em 18 pacientes (14,8%). Houve concordância entre os métodos para AAD HLA classe I (kappa = 0,66; p = 0,001) e HLA classe II (kappa = 0,54; p = 0,025)[25].

O esforço na obtenção de metodologias mais sensíveis para a detecção de anticorpos tem por objetivo final propiciar sobrevivência prolongada do enxerto, auxiliando na identificação e manejo da sensibilização imunológica dos receptores renais e principalmente avaliando a segurança do procedimento do transplante.

3. MARCO CONCEITUAL

A complexidade da avaliação de doadores e receptores está demonstrado na figura 7. Objeto desse estudo, as novas ferramentas diagnósticas são correlacionadas e refinam a avaliação imunológica no período pré-transplante renal em conjunto com as condições clínicas dos receptores e doadores.

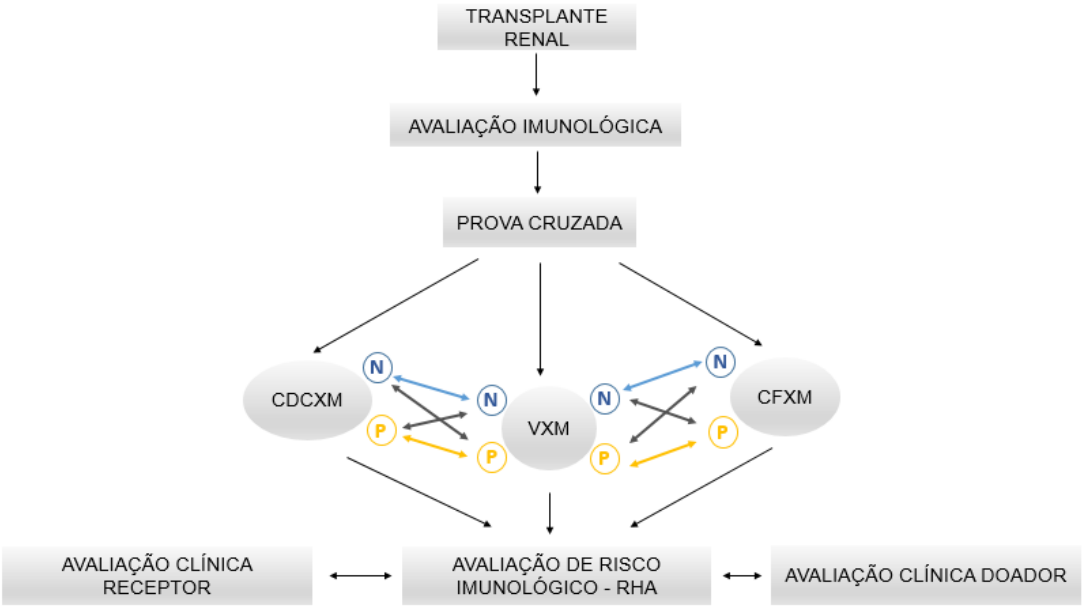


Figura 7: Marco conceitual da avaliação imunológica pré transplante renal. Elaborada pela autora.

Legenda: CDCXM, prova cruzada por citotoxicidade dependente de complemento; VXM, prova cruzada virtual; CFXM, prova cruzada por citometria de fluxo; RHA, rejeição humoral aguda; N, teste negativo; P, teste positivo.

4. JUSTIFICATIVA

A acurácia dos testes diagnósticos utilizados na seleção do receptor para transplante renal impacta diretamente nas condutas clínicas pré-transplante e nos desfechos clínicos. Além disso, avaliar o impacto de novas metodologias na prática clínica utilizando-as como teste único agrega um conhecimento que poderá contribuir para uma maior sobrevida do paciente e do enxerto.

5. OBJETIVOS

5.1. Objetivo Geral

O objetivo geral do estudo foi avaliar clínico-laboratorialmente os novos protocolos para a avaliação imunológica pré-transplante renal.

5.2. Objetivos Específicos

5.2.1. Estabelecer a correlação laboratorial entre os diferentes métodos de avaliação imunológica pré-transplante renal, retrospectivamente em provas cruzadas.

5.2.2. Acompanhar a evolução clínica e laboratorial dos pacientes submetidos a transplante avaliados no pré-operatório unicamente com CFXM pelo protocolo HALIFAX.

6. REFERÊNCIAS BIBLIOGRÁFICAS

1. Garcia CD, Pereira JD, Garcia VD. Doação e transplante de órgãos e tecidos. Brasil2015.

2. Interativa HM. Registro Brasileiro de Transplantes de 2017. 2018.

3. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. N Engl J Med. 1969;280(14):735-9.

4. Tinckam K. Histocompatibility methods. Transplant Rev (Orlando). 2009;23(2):80-93.

5. Kumbala D, Zhang R. Essential concept of transplant immunology for clinical practice. World J Transplant. 2013;3(4):113-8.

6. Tait BD, Susal C, Gebel HM, Nickerson PW, Zachary AA, Claas FH, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. Transplantation. 2013;95(1):19-47.

7. Gebel HM, Bray RA. Laboratory assessment of HLA antibodies circa 2006: making sense of sensitivity. Transplantation Reviews. 2006;20(4):189-94.

8. Terasaki PI. A personal perspective: 100-year history of the humoral theory of transplantation. Transplantation. 2012;93(8):751-6.

9. Hickey MJ, Valenzuela NM, Reed EF. Alloantibody Generation and Effector Function Following Sensitization to Human Leukocyte Antigen. Front Immunol. 2016;7:30.

10. Abbas AK, Lichtman AH, Pillai S. IMUNOLOGIA CELULAR E MOLECULAR. 7ª ed2012.

11. Bartel G, Schwaiger E, Bohmig GA. Prevention and treatment of alloantibody-mediated kidney transplant rejection. Transpl Int. 2011;24(12):1142-55.

12. Racusen LC, Halloran PF, Solez K. Banff 2003 meeting report: new diagnostic insights and standards. Am J Transplant. 4. United States2004. p. 1562-6.

13. Malheiro J, Centro Hospitalar do Porto P, Portugal, Tafulo S, Instituto do Sangue e Transplantação P, Portugal. Clinical implications of anti-HLA antibodies testing in kidney transplantation. Port J Nephrol Hypert. 2018;32(1):42-51.

14. Bontadini A. HLA techniques: typing and antibody detection in the laboratory of immunogenetics. Methods. 2012;56(4):471-6.

15. Ayna TK, Soyoz M, Kurtulmus Y, Dogan SM, Ozyilmaz B, Tugmen C, et al. Comparison of complement-dependent cytotoxic and flow-cytometry crossmatch results before cadaveric kidney transplantation. Transplant Proc. 2013;45(3):878-80.

16. Bray RA. Lymphocyte crossmatching by flow cytometry. Methods Mol Biol. 2013;1034:285-96.

17. Liwski RS, Gebel HM. Of Cells and Microparticles: Assets and Liabilities of HLA Antibody Detection. Transplantation. 2018;102(1S Suppl 1):S1-s6.

18. Liwski R, Adams G, Peladeau G, Eckels D, Bray R, Gebel H. 45-P An optimized flow cytometric crossmatch assay expedites pre-transplant immunologic risk assessment. Human Immunology. 2011;72:S48.

19. Liwski RS, Greenshields AL, Conrad DM, Murphey C, Bray RA, Neumann J, et al. Rapid optimized flow cytometric crossmatch (FCXM) assays: The Halifax and Halifaster protocols. Hum Immunol. 2018;79(1):28-38.

20. Wong G, Teixeira-Pinto A, Chapman JR, Craig JC, Pleass H, McDonald S, et al. The Impact of Total Ischemic Time, Donor Age and the Pathway of Donor Death on Graft Outcomes After Deceased Donor Kidney Transplantation. Transplantation. 2017;101(6):1152-8.

21. Bingaman AW, Murphey CL, Palma-Vargas J, Wright F. A virtual crossmatch protocol significantly increases access of highly sensitized patients to deceased donor kidney transplantation. Transplantation. 2008;86(12):1864-8.

22. Karpinski M, Rush D, Jeffery J, Exner M, Regele H, Dancea S, et al. Flow cytometric crossmatching in primary renal transplant recipients with a negative anti-human globulin enhanced cytotoxicity crossmatch. J Am Soc Nephrol. 2001;12(12):2807-14.

23. Ho EK, Vasilescu ER, Colovai AI, Stokes MB, Hallar M, Markowitz GS, et al. Sensitivity, specificity and clinical relevance of different cross-matching assays in deceased-donor renal transplantation. Transpl Immunol. 2008;20(1-2):61-7.

24. Wu P, Jin J, Everly MJ, Lin C, Terasaki PI, Chen J. Impact of alloantibody strength in crossmatch negative DSA positive kidney transplantation. Clin Biochem. 2013;46(15):1389-93.

25. Gil BC, Kulzer ASS, de Moraes P, Toresan R, da Rosa Vicari A, Dos Santos Fagundes I, et al. Comparative analysis of two methods to detect donor-specific anti-HLA antibodies after kidney transplant. Transpl Immunol. 2018.

26. Sullivan HC, Liwski RS, Bray RA, Gebel HM. The Road to HLA Antibody Evaluation: Do Not Rely on MFI. Am J Transplant. 2017;17(6):1455-61

7. PRODUÇÃO CIENTÍFICA

7.1 Capítulo de livro

7.1.1 Doação de Órgãos e Tecidos, 2015**.**

(16) Avaliação Imunológica Pré-Transplante.

Jorge Milton Neumann, Jamile Abud, Leticia Thais Nogueira, Yuri Thomé Machado Strey.

7.1.2 Laboratório na Prática Clínica, 2016.

(7) Fatores Interferentes em Análises Clínicas.

Jamile Abud, Jorge Miguel Dora, Luciana Scotti.

7.2 Trabalhos publicados em anais de eventos

7.2.1 Protocolo Halifax para Prova Cruzada por Citometria de Fluxo: Economia de Tempo sem Perda de Qualidade, 2014.
Juliana Montagner, Jamile Abud, Jorge Neumann

XII Congresso Português de Transplantação, XIII Congresso Luso Brasileiro de Transplantação, I Encontro Ibérico de Transplantação.

7.2.2 Avaliação dos desfechos do transplante renal em pacientes selecionados para transplante com doadores falecidos pela prova cruzada por citometria de fluxo utilizando o protocolo HALIFAX, 2015.
Jamile Abud, Juliana Montagner, Bruna Brasil Dal Pupo, Elizete Keitel, Roberto Manfro e Jorge Neumann.

XIV Congresso Brasileiro de Transplantes 2015.

7.2.3 Determinação da Frequencia de Alelos HLA -A, -B, -C, -DRB1 e DQ1 em Doadores Falecidos de Órgãos Sólidos, 2016.
Jamile Abud, Jessica Roman Ross, Heloisa Tarasconi, Julia Poeta e Jorge Neumann.

XIII Congresso Português de Transplantação, XV Congresso Luso Brasileiro de Transplantação, II Encontro Ibérico de Transplantação.

7.2.4 Crossflow no Pré Transplante: Desfechos Clínicos e Laboratoriais em Pacientes Transplantados Renais, 2016 comunicação breve.
Jamile Abud, Bruna Brasil Dal Pupo, Cyntia Keitel, Roberto Manfro e Jorge Neumann.

XIII Congresso Português de Transplantação, XV Congresso Luso Brasileiro de Transplantação, II Encontro Ibérico de Transplantação.

7.2.5 Impacto dos Anticorpos Anti-HLA pré-formados na sobrevida do enxerto renal: Experiência de um centro brasileiro, 2017.
Cynthia Keitel, Gisele Meinerz, Rosana MussoiBruno, Jamile Abud, Juliana Montagner, Damaris M.B Dorsdt, André K. Coutinho, Jorge Neumann, Valter Duro Garcia, Elizete Keitel.

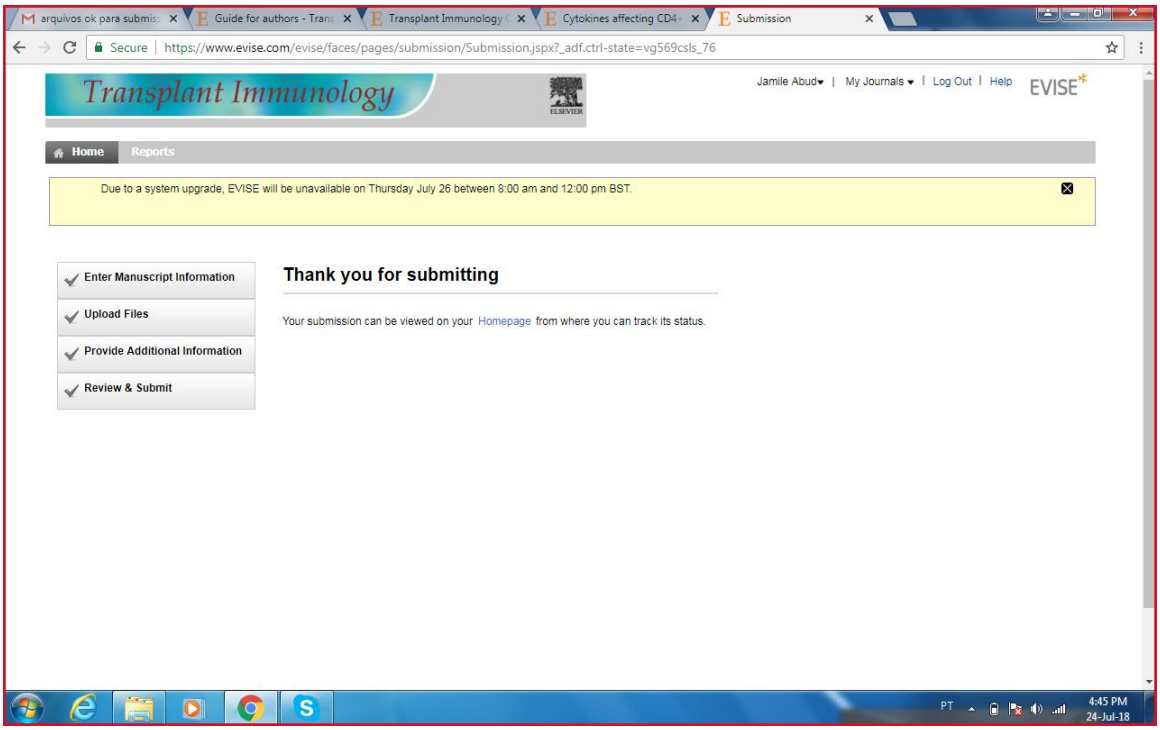
XV Congresso Brasileiro de Transplantes 2017

7.2.6 Crossflow Protocolo Halifax Diminui a Incidência de Rejeição Aguda Mediada por Anticorpos? 2017
Jamile Abud, Bruna Brasil Dal Pupo, Cynthia Keitel, Elizete Keitel, Valter Duro Garcia, Roberto Manfro, Jorge neumann.

XV Congresso Brasileiro de Transplantes 2017

7.3 Manuscritos

7.3.1 Phasing out the pre-transplant cytotoxicity crossmatch: Is something being lost?
Artigo original em inglês submetido à revista *Transplant Immunology* em 24/07/2018.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Phasing out the pre-transplant cytotoxicity crossmatch: Is something being lost?

Abbreviated title: Flow and cytotoxicity crossmatch

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Conflict of Interest: None.

ABSTRACT

The anti-human globulin-enhanced complement-dependent cytotoxicity crossmatch (AHG-CDCXM) assay has been used to assess the presence of donor-specific antibodies (DSA) in recipient's serum before kidney transplantation. The flow cytometric crossmatch (FCXM) assay was first introduced as an additional test. This study compared the outcomes of a cohort of kidney transplant patients that underwent FCXM only (FCXM group) versus a cohort of kidney transplant patients that underwent AHG-CDCXM (control group). The aim was to clinically validate the single use of the FCXM assay. Ninety-seven patients in the FCXM group and 98 controls were included. All crossmatches in the control group were negative. One patient in the FCXM group had a positive B cell crossmatch. One year after transplantation, there were no significant differences in patient survival (p = 0.591) and graft survival (p = 0.692) between the groups. Also, no significant difference was found in the incidence of Banff ≥ 1A acute cellular rejection episodes (p = 0.289). However, acute antibody-mediated rejections occurred in 3 controls (p = 0.028). The results showed that discontinuing the AHG-CDCXM assay do not modify the clinical outcomes in a 1-year follow-up.

Keywords: flow cytometric crossmatch, complement-dependent cytotoxicity crossmatch, acute cellular rejection, transplantation

Abbreviations

ABMR	antibody-mediated rejection
AHG-CDCXM	anti-human globulin-enhanced complement-dependent cytotoxicity crossmatch
CDCXM	complement-dependent cytotoxicity crossmatch
ECD	expanded criteria donor
FCXM	flow cytometric crossmatch
HLA	human leukocyte antigen
PCR	protein-to-creatinine ratio
PRA	panel-reactive antibody
SPA	solid phase assays

1. INTRODUCTION

Pre-transplant immunologic risk assessment is a key element in the clinical selection of potential recipients for a deceased donor kidney transplant. Sensitive and accurate tools for early detection of HLA antibodies in recipient serum, such as solid phase assays (SPA), allow the prediction of crossmatch results and help to guide the use of immunosuppressive agents in the presence of donor-specific antibodies (DSA) [1]. Nonetheless, the B and T cell crossmatch remains essential to decision-making for transplantation in most centers [2].

The complement-dependent cytotoxicity crossmatch (CDCXM) assay was proposed by Terasaki in 1969 [3,4] and has been commonly used to assess donor-recipient antibodies [5]. Since then, modifications have been made to enhance its sensitivity, such as the addition of anti-human globulin (AHG), as some patients had no detectable antibodies on the CDCXM but suffered from acute antibody-mediated graft rejection and graft loss [6]. A substantial increase in crossmatch sensitivity was observed with the use of the flow cytometric crossmatch (FCXM) [7,8]. Not only did the FCXM assay provide enhanced sensitivity but also required less time to be performed, leading to a reduction in cold ischemia time (CIT), which is inherent to deceased donor transplantation and one of the main predictors of initial graft function [9]. In 2011, a new FCXM protocol was proposed by Liwski et al. [10] The so-called Halifax protocol reduced even further the total assay time, thereby contributing to a significant decrease in CIT.

In this context, our laboratory adopted the Halifax FCXM protocol as the single pre-transplant crossmatch assay in September 2013. The present study assessed the clinical and laboratory outcomes in kidney transplant patients who underwent pre-transplant immunologic risk assessment with a single FCXM compared with patients from the period when CDCXM were used. The aim was to clinically validate the single use of the FCXM assay in the decision-making process for transplantation, and also to

assess if the lack of information regarding complement fixing antibodies, the CDC crossmatch, could have any negative impact on our transplants.

2. PATIENTS AND METHODS

2.1. Patients

We followed a cohort of 100 kidney transplant patients who were selected consecutively and assessed with a single FCXM before transplantation (FCXM group). Similarly, we studied a retrospective cohort of 100 kidney transplant patients who were assessed with the CDCXM assays (control group).

Adult and pediatric patients who received a kidney transplant from deceased donors from the state of Rio Grande do Sul, Brazil, were included in the study. The post-transplant follow-up period was 1 year.

2.2. Immunologic risk assessment

The result of the panel-reactive antibody (PRA) tests performed in the patients' sera in the last four months before transplantation was collected. Single-antigen bead (SAB) assays (LABScreen Single Antigen Beads, OneLambda, CA, USA) were performed in all recipients. The SAB protocol included heat treatment of the sera tominimize false-negative reactions. PRA scores for HLA class I and II antibodies were used, as well as specificity and mean fluorescence intensity (MFI) of HLA class I and II antibodies when these were present. The tests were conducted according to the manufacturer's instructions, and the Luminex 100 system and the Fusion HLA software were used to analyze the results. The antibodies were considered positive if the MFI was higher than 1,000 and we considered DSA for HLA-A, -B and DRB1 for all patients. In patients typed for HLA-C and HLA-DQB1, these antibodies were also considered.

HLA typing of donors (HLA-A, -B, -C, -DRB1, -DQB1) and recipients (HLA-A, -B, -DRB1 in all and HLA-C and DQB1 in some) was performed by a sequence-specific

primer set (SSP, One Lambda, CA, USA) according to the manufacturer's instructions. The number of donor-recipient HLA mismatches were analyzed based on HLA typing for HLA-A, HLA-B, and HLA-DRB1.

Donor lymph nodes or spleen were used as sources of cells to perform the FCXM and CDCXM assays with the two latest recipient sera, stored at -80°C. Cells were separated by Ficoll-Hypaque density gradient centrifugation. The FCXM assay was conducted according to the Halifax protocol. Pronase treatment of lymphocytes was done [11], and T and B cells were assessed using peridinin-chlorophyll-protein complex (PERCP) anti-human CD3 (clone SK7, BD Biosciences) and phycoerythrin (PE) anti-human CD19 (clone HIB19, BD Biosciences). Fluorescein isothiocyanate (FITC) F(ab')2 Anti-Human IgG, Fc fragment specific (Jackson Immuno Research Laboratories, USA) was added. The samples were collected and analyzed with the BD FACSCalibur flow cytometer (BD Biosciences), and cut-off scores were set at 40 for T cells and 100 for B cells. The CDCXM plus anti-human globulin (AHG-CDCXM) assay was performed for T-cells and CDCXM not modified was performed for B cells. The protocols conducted according to the American Society for Histocompatibility and Immunogenetics (ASHI) protocol [12] using a fluorescent marker for dead cell quantification and magnetic beads for T and B cell separation.

2.3. Clinical and predictive variables

Demographic data of donors and recipients were collected. Donors were classified as expanded criteria donors (ECD) according to the definition of the United Network for Organ Sharing (UNOS). Data on CIT, underlying diseases, and previous transplantation were collected from the recipients' electronic medical records. In the study period, there were no changes in the immunosuppression protocols of the transplantation center. All DSA negative transplant patients were treated with the anti-CD25 monoclonal antibody (interleukin-2 receptor). Patients with PRA score higher than 50%, DSA positive patients, and patients whose donors had CIT higher than 24

hours were treated with anti-thymocyte globulin (ATG). The maintenance therapy consisted of tacrolimus, mycophenolate, and prednisone.

2.4. Clinical outcomes

Protein-to-creatinine ratio (PCR) and estimated glomerular filtration rate (eGFR) were evaluated at 3, 6, and 12 months after transplantation. eGFR was calculated by the Modification of Diet in Renal Disease (MDRD) Study equation [13,14] in adult patients and by the Schwartz equation in patients younger than 18 years old [15]. In patients who underwent post-transplant SAB testing, the presence of *de novo* DSA was determined. Delayed graft function (DGF) was defined as the need for dialysis until the seventh day after transplantation, and DGF length was measured. Rejections were categorized based on the interpretation of the transplant pathologist according to the Banff 2007 classification [16]. Acute antibody-mediated rejection (ABMR) was assessed according to the Banff 2013 classification [17]. Graft loss was defined as the need to resume dialysis, and the causes of graft loss were collected from medical records. Deaths and their causes were collected from medical records and reviewed by a physician from the kidney transplantation team.

2.5. Statistical analysis and ethical aspects

StatCalc and SPSS, version 20, were used for the statistical analyses. Categorical variables were expressed as absolute frequencies (number of patients) and relative frequencies (percentage). Parametric data were compared using the Student's t-test, while non-parametric data were compared using the Mann-Whitney U test. Categorical variables were compared using the chi-square test. The Kaplan-Meier survival analysis was used for patients and grafts. Multivariate analyses not were done. For statistical purposes, a significance level below 0.05 was set. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist was used to

guide the study. This study was approved by the Research Ethics Committee of Santa Casa Hospital from Porto Alegre, state of Rio Grande do Sul, southern Brazil, under protocol number 41095914.1.0000.5335.

3. RESULTS

3.1. Donor and recipient characteristics at the time of transplant

We assessed 97 patients in the FCXM group (kidney transplants performed between October 2013 and October 2014) and 98 patients in the control group (kidney transplants performed between October 2012 and September 2013). Five patients were excluded because of combined liver-kidney transplants. Pre-transplant demographic and clinical data of the groups are shown in Table 1.

Deceased donors were all brain dead. There were 116 male donors overall, including 46 in the FCXM group (47.4%) and 70 in the control group (71.4%) ($p<$ 0.001). Mean donor age was 41±18.8 years in the FCXM group and 40±21.4 years in the control group ($p = 0.124$). Thirty-eight donors in the FCXM group (40%) and 39 controls (39.8%) were classified as ECD, with no significant between-group difference ($p = 0.977$). CIT did not differed between the groups (FCXM group: 20.4±5.5hours; control group: 20.3±4.9 hours; $p = 0.342$). HLA-A, -B, and -DRB1 types were available for all donors, while HLA-C and -DQB1 types were typed in 60.5% ($n = 118$) and 63.5% ($n = 124$) of the donors, respectively. No donor was genotyped for HLA-DPB1.

There were no significant differences between mean PRA scores for anti-HLA class I antibodies (FCXM group: 21.0%±31.0; control group: 17.2%±29.3; $p = 0.427$) and anti-HLA class II antibodies (FCXM group: 13.9%±22.6; control group: 15.1%±24.4; $p = 0.315$). DSA, either class I or II, were absent in 86% of the patients before transplantation. Pre-transplant PRA scores are shown in Table 1. The mean number of mismatches for the HLA-A, -B, and -DRB1 loci was 4 ±1 ($p = 0.542$) in both groups.

Table 1. Baseline characteristics of kidney transplants in the studied groups.

Variables	Total number of patients	FCXM group (n=97)	Control group (n=98)	*p*
Donors				
Male – *n* (%)	195	46 (47.4)	70 (71.4)	0,01
Female – *n* (%)		51 (52.6)	28 (28.6)	
Age - years; mean (SD)	195	41 (18.8)	40 (21.4)	0.124
Recipients				
Male – *n* (%)	195	60 (61.9)	52 (53.1)	0.273
Female – *n* (%)		37 (38.1)	46 (46.9)	
Age at transplant – years; mean (SD)	195	44 (18.3)	45 (19.7)	0.653
Primary kidney disease – *n* (%)	195			0.09
Unknown		48 (49.0)	33 (34.4)	
Hypertension		10 (11.0)	26 (27.1)	
Diabetes		15 (16.4)	15 (15.6)	
Polycystic kidney disease		9 (9.9)	5 (5.2)	
Others		17 (18.7)	17 (17.6)	
Number of kidney transplants – *n* (%)	195			0.833
First		80 (82.5)	79 (80.7)	
Second or third		17 (17.5)	19 (19.3)	

Table 1: (continued)

Class I DSA – *n* (%)	195			0.803
Absent		83 (85.6)	83 (84.7)	
1		9 (9.3)	12 (12.2)	
2		3 (4.1)	3 (3.1)	
3		1 (1.0)	-	
Class II DSA – *n* (%)	195			0.277
Absent		84 (86.6)	92 (93.9)	
1		9 (9.3)	5 (5.1)	
2		3 (3.1)	1 (1.0)	
3		1 (1.0)	-	
DSA MFI-Sum – median (IQR)				
Class I (FCXM group n=14; control group n=15)	29	2,342 (1,203-3,408)	2,669 (1,794-3,845)	0.270
Class II (FCXM group n=13; control group n=06)	19	3,359 (1,462-8,436)	1,838 (1,212-6,553)	0.467

DSA MFI-Sum, the sum of the mean fluorescence intensity of the different circulating donor-specific antibodies; FCXM, flow cytometric crossmatch.

3.2. Clinical and immunological outcomes

One hundred and fifteen recipients (59%) presented DGF, with no significant difference between the groups (Table 2). Estimated GFR and urinary protein excretion (total protein/creatinine in a urine sample) were assessed at 3, 6, and 12 months after transplantation. Urinary PCRs were available from 128 patients at 3 and 6 months post-transplant and 127 patients at 12 months post-transplant. eGFR analysis was made separately for patients aged less than 18 years and patients aged 18 years or older. No differences in eGFR were observed over time in the group of recipients younger than 18 years old. In adult recipients, a significantly higher eGFR was observed in the FCXM group at 12 months after transplantation (Table 2).

Table 2. Graft function outcomes.

Variables	Number of patients evaluated	FCXM group	Control group	*p*
DGF – *n* (%)	194	59 (60.8)	56 (57.7)	0.770
DGF, days – mean (SD)		5.5 (4.0)	4.58 (3.5)	0.280
PCR, median (IQR)				
3 months	128	0.30 (0.2-0.5)	0.37 (0.2-0.5)	0.120
6 months	128	0.28 (0.2-0.5)	0.36 (0.2-0.7)	0.240
12 months	127	0.23 (0.2-0.5)	0.33 (0.2-0.6)	0.150
eGFR< 18 years (mL/min/1.73m^2), median (IQR)				
3 months	21	65 (53-72)	70 (65-75)	0.223
6 months	21	65 (57-68)	65 (60-75)	0.605
12 months	20	81 (54-103)	65 (55-71)	0.412
eGFR> 18 years (mL/min/1.73m^2), median (IQR)				
3 months	157	38.0 (29.5-49.0)	37.5 (17.0-51.0)	0.267
6 months	146	43.5 (29.0-54.3)	40.0 (15.0-50.8)	0.090
12 months	128	46.0 (33.0-57.0)	39.0 (22.5-49.0)	0.009

DGF, delayed graft function; eGFR, estimated glomerular filtration rate; FCXM, flow cytometric crossmatch; PCR, protein-to-creatinine ratio.

All T and B cell crossmatches were negative in the control group. In the FCXM group, one patient presented a positive B cell FCXM with a 193 channel shift. The positive finding was attributed to an anti-HLA-DQ6 DSA. The recipient underwent a prior kidney transplant, had Banff type IB acute cellular rejection and maintained a functioning graft at the end of the follow-up period.

Sixteen patients from the FCXM group underwent a clinically indicated SAB test in a mean post-transplant time of 82.0±22.9 days, while 18 controls underwent the same test in a mean post-transplant time of 87.0±19.5 days (p = 0.852). In the FCXM group, 4 patients had class I DSA and 3 had class II DSA, while in the control group, 7 patients had class I DSA and 4 had class II DSA (Table 3).

Table 3. Presence of DSA after transplantation.

DSA	**FCXM group (*n* = 16)**	**Control group (*n* = 18)**	***p***
Class I – *n* (%)			0.558
Absent	12 (75)	10 (55.6)	
1	2 (12.5)	3 (16.7)	
2	1 (6.3)	1 (5.6)	
3	1 (6.3)	4 (22.2)	
Class II – *n* (%)			0.942
Absent	13 (81.3)	14 (77.8)	
1	2 (12.5)	3 (16.7)	
2	1 (6.3)	1 (5.6)	
De novo DSA – *n* (%)	5 (5.2)	8 (8.2)	0.400

DSA, donor-specific antibodies; FCXM, flow cytometric crossmatch.

3.3. Survival analysis

One year after transplantation, there were no significant differences in patient survival (FCXM group: 92.8%; control group: 90.8%; $p = 0.591$) and graft survival (FCXM group: 84.5%; control group: 82.7%; $p = 0.692$) (Figures 1 and 2). Sixteen patients died in the follow-up period, 7 in the FCXM group and 9 in the control group ($p = 0.811$), most of them ($n = 11$) due to infections. There were 15 (15.5%) graft losses in the FCXM group and 17 (17.3%) in the control group, with no significant between-group difference ($p = 0.872$). Two failures were caused by antibody-mediated rejection in the control group, while no graft loss due to immunological causes was observed in the FCXM group.

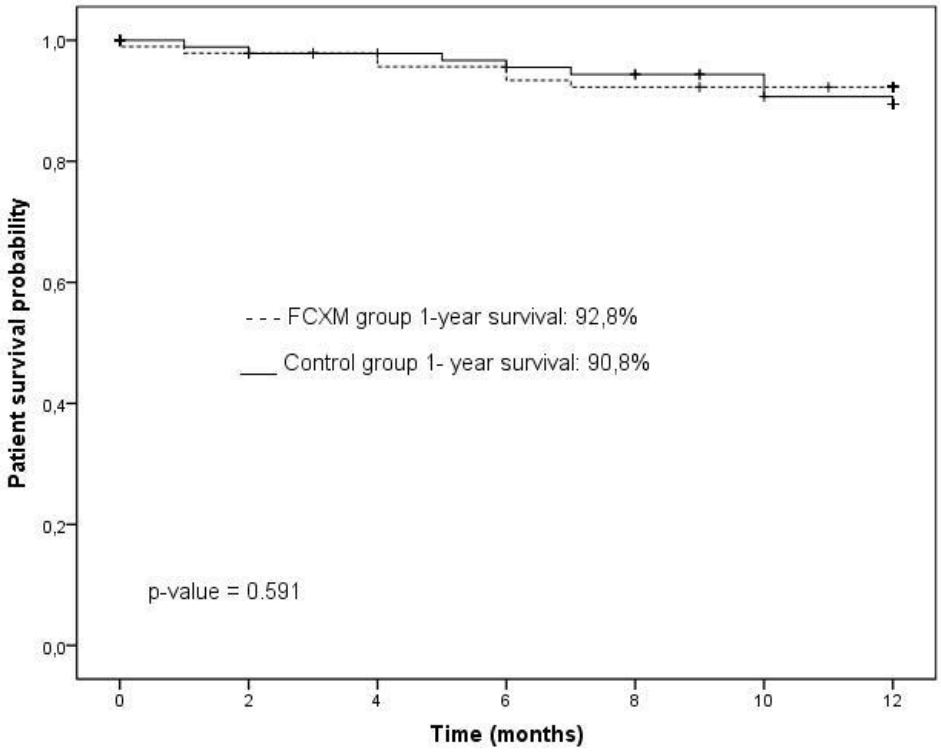

Figure 1. Patient survival rate.

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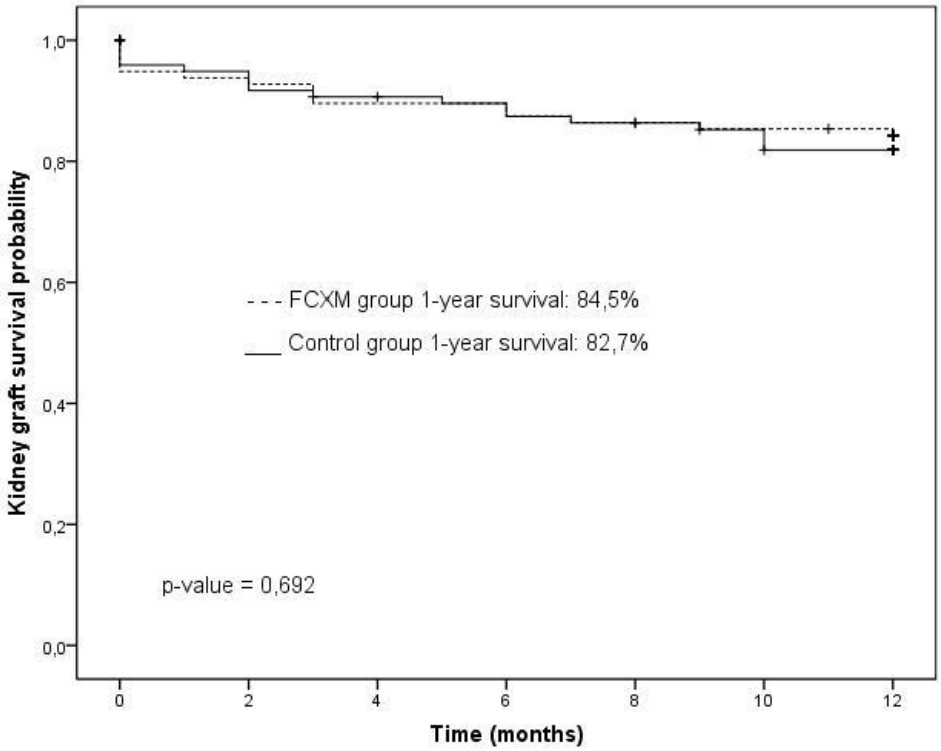

Figure 2. Graft survival rate.

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3.4. Graft biopsy data

Sixty-three (48%) patients in the FCXM group and 68 (52%) controls underwent a kidney graft biopsy. As shown in Table 4, no significant difference was found in the incidence of acute cellular rejection equal to or greater than 1A [16] (p = 0.289). However, acute ABMR occurred in 3 patients in the control group and none in the FCXM group (p = 0.04). In the 3 patients with ABMR, none had pre transplant DSA, all received grafts from ECD donors, had DGF and formed *denovo* DSAs, two lost the graft and none died. C4d deposits along peritubular capillaries were absent in 47 (85.5%) and 30 (49.2%) patients in the FCXM group and the control group, respectively. Any level of C4d intensity was detected in 8 (14.5%) and 31 (50.8%) patients in the FCXM group and the control group, respectively (p< 0.001).

Table 4. Histopathologic findings in graft biopsy.

Pathology test results – *n* (%)	FCXM group (*n* = 63)	Control group (*n* = 68)	*p*
No rejection	16 (25.4)	20 (29.4)	0.750
Borderline rejection	10 (15.9)	13 (19.1)	0.638
Acute cellular rejection ≥ 1A	27 (42.9)	21 (30.8)	0.293
Acute antibody-mediated rejection	-	3 (4.5)	0.028
Interstitial fibrosis and tubular atrophy	1 (1.6)	5 (7.4)	0.246
Other findings	9 (14.3)	6 (8.8)	0.667

FCXM, flow cytometric crossmatch.

4. DISCUSSION

In the present study we assessed the FCXM as the only crossmatch assay in patients undergoing a deceased donor kidney transplant. No significant differences were found in the main clinical outcomes of the group that underwent FCXM alone compared with the group that underwent AHG-CDCXM (controls).

There were no statistically significant differences in the incidence of DGF and urinary PCR one year after transplantation. eGFR was higher in the FCXM group than in the control group. Among those recipients who underwent clinically indicated SAB testing, most patients from both groups did not develop DSA one year after transplantation. Importantly, patient and graft survivals were not significantly different between the groups. The incidence of acute cellular rejection was not different between groups. However, three cases of acute ABMR were observed in the control group compared with none in the FCXM group.

The studied groups were homogeneous in terms of risk predictors (donor age, underlying disease, CIT, number of HLA mismatches, pre-transplant PRA score, and DSA screening), which contributed to reducing biases in the analyses. In order to reduce variability in organ quality, transplants of organs coming from other Brazilian

states were not included. In both groups, the presence of DSA was evaluated through SAB assay before transplantation.

In a 2008 study of 354 kidney transplant patients, Ho et al. [18] evaluated the sensitivity and specificity of the CDCXM, FCXM, and SPA assays using graft loss as the main outcome. These three tests were performed in all patients to assess the presence of DSA. There was no significant difference in graft survival between these methods in a 3-year follow-up for both first transplant and re-transplant patients. The authors reported the importance of the CDCXM and FCXM assays according to each method's sensitivity. Their results are consistent with our findings, although the two studies were designed differently, as the FCXM assay was used as the single crossmatch method in our study. In consonance with our findings a former retrospective U.S. study examined survival and clinical outcomes in 624 kidney transplant patients, mostly from deceased donors, tested only with the FCXM assay and divided into three groups (T^- B^- FCXM, T^- B^+ FXCM, and T^+ B^+ FXCM), and reported a 1-year graft survival of 90% in the T^- B^- FCXM group [19–21].

Unlike the AHG-CDCXM assay, the FCXM assay stratifies the risk and might not necessarily contraindicate transplantation when the result is positive. Graff et al. in 2009 studied retrospectively the outcome implications of positive FCXM results, using data for a national cohort of transplant recipients recorded in the Organ Procurement and Transplant Network registry data. They observed a continued detrimental effect of a positive FCXM result beyond the first transplant anniversary [22]. We had one recipient transplanted after B^+ FCXM in the FCXM group and this patient is free of dialysis three years after transplantation.

Our laboratory used to perform both the AHG-CDCXM and FCXM assays by the standard ASHI protocol. The Halifax protocol encouraged us to adopt the FCXM assays as the sole crossmatching evaluation. This strategy allowed a reduction in the time required to perform the test. However, contrary to our expectation, the CIT did not

decrease. This can be explained by the fact that the process of organ donation involves multiple teams and factors that are independent of the crossmatch assay.

This study has some limitations. Firstly, it is a single-center, non-randomized study with a retrospective control group. We believe that this limitation did not impact the results as the overall practice, including immunosuppressive regimens, was essentially the same throughout the study period. Secondly, post-transplant DSA results were not available for all recipients, and HLA-C and DQB1 loci were not typed for all recipients. However, the number of recipients with post-transplant SAB testing was similar between groups suggestion a similar clinical need for such testing in clinical practice.

5. CONCLUSIONS

The sensitivity of the methods used to detect HLA class I and II antibodies have constantly been increased as a result of advances in tests such as the FCXM and SPA assays. The main purpose of the present study was to demonstrate that discontinuing the use of the AHG-CDCXM assay does not modify the clinical outcome on the kidney transplants setting**.** A combined assessment using the SAB test and the FCXM assay should be performed to evaluate risks and help the decision-making process [1]. Even though the higher sensitivity of the FCXM is well recognized this method is seldom used alone outside North America. Therefore we believe that validating its clinical application, by reporting our experience, could be a contribution to the field. Also important, the FCXM assay is far from standardized. Only recently a proposed standard protocol, the Halifax, was published [11]. Finally, it is important that each center validate their FCXM results with respect to acceptable clinical risks [1].

We are confident that the results described here strongly support the safety of using the Halifax FCXM as the only pre-transplant crossmatch test. And importantly, the lack of information regarding complement fixing antibodies, as in the CDCXM

assays, does not have a detrimental impact on the quality of kidney transplantation in our practice.

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REFERENCES

[1] B.D. Tait, C. Süsal, H.M. Gebel, P.W. Nickerson, A.A. Zachary, F.H.J. Claas, E.F. Reed, R.A. Bray, P. Campbell, J.R. Chapman, P.T. Coates, R.B. Colvin, E. Cozzi, I.I.N. Doxiadis, S. V. Fuggle, J. Gill, D. Glotz, N. Lachmann, T. Mohanakumar, N. Suciu-Foca, S. Sumitran-Holgersson, K. Tanabe, C.J. Taylor, D.B. Tyan, A. Webster, A. Zeevi, G. Opelz, Consensus guidelines on the testing and clinical management issues associated with HLA and Non-HLA antibodies in transplantation, Transplantation. 95 (2013) 19–47. doi:10.1097/TP.0b013e31827a19cc.

[2] K. Tinckam, Histocompatibility methods, Transplant. Rev. 23 (2009) 80–93. doi:10.1016/j.trre.2009.01.001.

[3] P.I. Terasaki, A personal perspective: 100-Year history of the humoral theory of transplantation, Transplantation. 93 (2012) 751–756. doi:10.1097/TP.0b013e3182483713.

[4] P.I. Patel, R., Teraski, Significance of the positive crossmatch test in kidney transplantation, N. Engl. J. Med. 280 (1969) 735–739.

[5] R.J. Graff, P.M. Buchanan, N. Dzebisashvili, M.A. Schnitzler, J. Tuttle-Newhall, H. Xiao, E. Schadde, A. Gheorghian, K.L. Lentine, The clinical importance of flow cytometry crossmatch in the context of CDC crossmatch results, Transplant. Proc. 42 (2010) 3471–3474. doi:10.1016/j.transproceed.2010.06.025.

[6] H.M. Gebel, R.A. Bray, Laboratory assessment of HLA antibodies circa 2006: making sense of sensitivity, Transplant. Rev. 20 (2006) 189–194. doi:10.1016/j.trre.2006.07.006.

[7] R.A. Bray, L.K. Lebeck, H.M. Gebel, The flow cytometric crossmatch. Dual-color analysis of T cell and B cell reactivities., Transplantation. (1989).

[8] G.H.M. Liwski, Robert S. Greenshields, Anna L. Bray, Robert A., Becoming a chef in the human leukocyte antigen kitchen: interpretation and modification of human leukocyte antigen-antibody assays, Curr. Opin. Organ Transplant. 22

(2017) 407–414.

[9] G. Wong, A. Teixeira-Pinto, J.R. Chapman, J.C. Craig, H. Pleass, S. Mcdonald, W.H. Lim, The impact of total ischemic time, donor age & the pathway of donor death on graft outcomes after deceased donor kidney transplantation, Transplantation. 101 (2017) 1152–1158. doi:10.1097/TP.0000000000001351.

[10] R.S. Liwski, Optimization of flow cytometric crossmatch assay to expedite pre-transplant immunologic risk assessment, in: Conf. Am. Soc. Histocompat. Immunogenet. Annu. Meet., 2013. doi:10.13140/2.1.4965.6962.

[11] R.S. Liwski, A.L. Greenshields, D.M. Conrad, C. Murphey, R.A. Bray, J. Neumann, H.M. Gebel, Rapid optimized flow cytometric crossmatch (FCXM) assays: The Halifax and Halifaster protocols, Hum. Immunol. 79 (2018) 28–38. doi:10.1016/j.humimm.2017.10.020.

[12] S.R.M. Hahn Amy B., Land Geoffrey A., American Society for Histocompatibility and Immunogenetics, ASHI Laboratory Manual, 2000.

[13] A.S. Levey, L.A. Stevens, C.H. Schmid, Y. Zhang, A.F. Castro III, H.I. Feldman, J.W. Kusek, P. Eggers, F. Van Lente, T. Greene, J. Coresh, A. Current Mailing address, V. Lente, Authors Current Mailing address: 8. Paul Eggers Kidney & Urology Branch, NIDDK, National Institutes of Health, 6707 Democracy Blvd, Ann Intern Med. 150 (2081) 604–612.

[14] A.S. Levey, J. Coresh, T. Greene, L.A. Stevens, Y. Zhang, S. Hendriksen, J.W. Kusek, F. Van Lente, Using Standardized Serum Creatinine Values in the Modification of Diet in Renal Disease Study Equation for Estimating Glomerular Filtration Rate, Ann Intern Med. 145 (2006) 247–254. www.annals.org.

[15] G.J. Schwartz, A. Muñ, M.F. Schneider, R.H. Mak, F. Kaskel, B.A. Warady, S.L. Furth, New Equations to Estimate GFR in Children with CKD, J Am Soc Nephrol. 20 (2009) 629–637. doi:10.1681/ASN.2008030287.

[16] K. Solez, R.B. Colvin, L.C. Racusen, M. Haas, B. Sis, M. Mengel, P.F. Halloran, W. Baldwin, G. Banfi, A.B. Collins, F. Cosio, D.S.R. David, C. Drachenberg, G.

Einecke, A.B. Fogo, I.W. Gibson, D. Glotz, S.S. Iskandar, E. Kraus, E. Lerut, R.B. Mannon, M. Mihatsch, B.J. Nankivell, V. Nickeleit, J.C. Papadimitriou, P. Randhawa, H. Regele, K. Renaudin, I. Roberts, D. Seron, R.N. Smith, M. Valente, Banff 07 classification of renal allograft pathology: Updates and future directions, Am. J. Transplant. 8 (2008) 753–760. doi:10.1111/j.1600-6143.2008.02159.x.

[17] M. Haas, B. Sis, L.C. Racusen, K. Solez, D. Glotz, R.B. Colvin, M.C.R. Castro, D.S.R. David, E. David-Neto, S.M. Bagnasco, L.C. Cendales, L.D. Cornell, A.J. Demetris, C.B. Drachenberg, C.F. Farver, A.B. Farris, I.W. Gibson, E. Kraus, H. Liapis, A. Loupy, V. Nickeleit, P. Randhawa, E.R. Rodriguez, D. Rush, R.N. Smith, C.D. Tan, W.D. Wallace, M. Mengel, Banff 2013 meeting report: Inclusion of C4d-negative antibody-mediated rejection and antibody-associated arterial lesions, Am. J. Transplant. 14 (2014) 272–283. doi:10.1111/ajt.12590.

[18] E.K. Ho, E.R. Vasilescu, A.I. Colovai, M.B. Stokes, M. Hallar, G.S. Markowitz, V.D. D'Agati, D.J. Cohen, L.E. Ratner, N. Suciu-Foca, Sensitivity, specificity and clinical relevance of different cross-matching assays in deceased-donor renal transplantation, Transpl. Immunol. 20 (2008) 61–67. doi:10.1016/j.trim.2008.09.013.

[19] A.J. Norin, M.O. Mondragon-Escorpizo, A. Brar, D. Hochman, N. Sumrani, D.A. Distant, M.O. Salifu, Poor kidney allograft survival associated with positive B cell - Only flow cytometry cross matches: A ten year single center study, Hum. Immunol. 74 (2013) 1304–1312. doi:10.1016/j.humimm.2013.06.027.

[20] Registro Brasileiro de Transplantes de 2017, ABTO. (2017) 25. http://www.abto.org.br/abtov03/Upload/file/RBT/2017/rbt-imprensa-leitura-compressed.pdf (accessed May 31, 2018).

[21] A. Hart, J.M. Smith, M.A. Skeans, S.K. Gustafson, D.E. Stewart, W.S. Cherikh, J.L. Wainright, A. Kucheryavaya, M. Woodbury, J.J. Snyder, B.L. Kasiske, A.K. Israni, OPTN/SRTR 2015 Annual Data Report: Kidney, Am. J. Transplant. 17

(2017) 21–116. doi:10.1111/ajt.14124.

[22] R.J. Graff, H. Xiao, M.A. Schnitzler, P. Ercole, H. Solomon, T. Pessin, K.L. Lentine, The role of positive flow cytometry crossmatch in late renal allograft loss, Hum. Immunol. 70 (2009) 502–505. doi:10.1016/j.humimm.2009.04.002.

7.3.2. Accuracy of different methods used for crossmatch tests between decesead donor and renal receptors.

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Accuracy of different methods used for crossmatch tests between deceased donor and renal receptors.

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ABSTRACT

Background: Alloantibodies against donor human leukocyte antigens (HLA), named donor-specific antibodies (DSA) are one of the most important factors for graft survival. The complement-dependent microcytotoxicity crossmatch (CDCXM) is a standard technique to detect clinically significant DSA. Flow cytometric crossmatch (FCXM) and solid phase assays (SPA) were latter implemented and the transition from CDCXM to fluorescence-based detection systems aimed mainly to detect low levels of antibodies. Our objective was to quantify FCXM false positive results considering CDCXM and DSA results as the gold standard. *Methods:* 713 crossmatches were analyzed retrospectively by CDCXM, FCXM and Single Antigen Beads (SAB) among recipients waiting for a deceased donor kidney transplant in the year of 2010. All reactions were analyzed by two experts. SAB were considered positive when median fluorescence intensity was ≥1,000. *Results:* All CDCXM assays were positive in the FCXM (n=227; 31.83%). Among 486 (68.17%) negative CDCXM, 13 (1.82%) were FCXM positive and no DSAs were detected in the sera. The specificity of the FCXM for T and B cells were 88% and 87%, respectively. *Conclusions:* We found a low number of false positive cases considering the SAB test results. We consider that the three techniques are complementary, and ideally their applicability should be established according each transplantation center guidelines.

Abbreviations

AHG	anti-human globulin
AHGXM	anti-human globulin-enhanced complement-dependent cytotoxicity crossmatch
CDCXM	complement-dependent cytotoxicity crossmatch
DSA	donor-specific antibodies
FCXM	flow cytometric crossmatch
HLA	human leukocyte antigen
MFI	mean fluorescence intensity
PRA	panel-reactive antibody
SAB	single antigen beads
SPA	solid phase assays
VXM	virtual crossmatch

1. INTRODUCTION

Initially, histocompatibility tests should play a single and clear role in transplantation: avoid hyperacute rejection. For this goal, CDCXM was proposed by Terasaki in 1969 [1,2]. However, original CDCXM was not sensitive enough to detect low levels of antibodies [3]. Anti-human globulin (AHG) was latter added to the CDCXM to improve its sensitivity in order to detect lower antibodies anti-human leucocyte antigen (HLA) titers [4]. These tests have been commonly used to assess anti-donor antibodies in the recipient´s serum. After this step, the transplant community expected an increased graft survival. The flow cytometry crossmatch assay (FCXM) was introduced in the 1980s [5,6] as a more sensitive method involving a fluorescent secondary antibody. This is a quantification method and its result provides risk estimation rather than a contraindication for transplantation. More recently sensitive and accurate tools aiming at early detection of low levels HLA antibodies in recipient´s serum, such as solid phase assays (SPA), that identify antibody specificity, allowed the prediction of the crossmatch results, and is now called virtual crossmatch (VXM) [7].

The CDCXM and FCXM are the tests routinely used for evaluation of kidney transplant eligibility. The choice of which crossmatch test to perform represents a challenge to transplant programs. In this context, the present study analyzed FCXM false positive results considering CDCXM results and knowledge of the presence of DSA as the gold standard.

2. Materials and methods

2.1. Study Population

Adult and pediatric kidney transplant candidates registered in the waiting list for kidney transplantation at *Irmandade Santa Casa de Misericórdia* (ISCMPA) hospital, Porto Alegre, southern Brazil, were included in the study. We reviewed all crossmatches results performed at the final evaluation before kidney transplantation. A retrospective evaluation of CDCXM and FCXM crossmatches results and SPA assays performed at the Transplantation Immunology Laboratorywere collected over the year of 2010.

All patients were tested for T and B cells FCXM and the presence of DSA was accessed by Single Antigen Beads (SAB) for HLA classes I and II. Also, CDCXM for B cells and AHG enhanced CDCXM (AHGXM) for T cells were performed. We have either one or two crossmatches results for each donor-recipient pair and donor sera (most recent and historical, as available) was also tested by SAB for all pairs. The recipients' samples were stored at -80°C in the transplant immunology laboratory (LITx-ISCMPA) and the splenic or lymph nodes lymphocytes were obtained from the deceased donor. All results were reviewed by two experienced biomedical professionals.

2.2. CDCXM and AHGXM

CDCXM (B cells) and AHGXM (T cells) assays were conducted according to the American Society for Histocompatibility and Immunogenetics (ASHI) protocol [8] using a fluorescent marker for dead cell quantification and magnetic beads for T and B cell identification. A crossmatch was read as positive when cell lysis, detected by uptake of a vital dye, was ≥20% of that seen in negative control samples. We classified the results in four categories according the percentage of cell lysis: 2 (20 to 40%); 4 (41 to 60%); 6 (61 to 80%) and 8 (81 to 100%).

2.3. FCXM

Flow crossmatches tests for T and B cells was assessed using peridinin-chlorophyll-protein complex (PERCP) anti-human CD3 (clone SK7, BD Biosciences) and phycoerythrin (PE) anti-human CD19 (clone HIB19, BD Biosciences). Fluorescein isothiocyanate (FITC) F(ab')2 Anti-Human IgG, Fc fragment specific (Jackson Immuno Research Laboratories, USA) was added. The traditional flow crossmatch test was performed and the samples were collected and analyzed using BD FACS Calibur flow cytometer (BD Biosciences). Data was acquired using 10,000 channel scale and cut-off scores were set at 40 for T cells and 100 for B cells from negative control serum.

2.4. Presence of DSA

Results from the panel-reactive antibody (PRA) tests performed in patients' sera obtained at most four months before transplantation. SAB assays (LABScreen Single Antigen Beads, One Lambda, CA, USA) were performed in all recipients. The tests were conducted according to the optimized manufacturer's protocol, and analyzed by Luminex® 100 system. The Fusion HLA software was used to analyze the results. The antibodies were considered positive if the mean fluorescence intensity (MFI) was ≥1,000. SAB test was evaluated by a qualitative test approved by the Food and Drug Administration[9].

2.5. HLA typing

Donors were HLA-typed for A/B/DRB1 antigens by PCR-SSP by usinga sequence-specific primer set (SSP, One Lambda, CA, USA) according to the manufacturer's instructions, with low-resolution information.

2.6. Statistical Analyses

SPSS version 20 was used for statistical analyses. Categorical variables were expressed as absolute frequencies (number of patients) and relative frequencies (percentage). The prevalence-adjusted bias-adjusted kappa (Pabak) statistic test was used for analysis of agreement between methods.

The Standards for Reporting Diagnostic accuracy studies (STARD) checklist was used to guide the study. This study was approved by the Research Ethics Committee of *Santa Casa* Hospital from Porto Alegre, State of Rio Grande do Sul, under protocol number 41095914.1.0000.5335.

3. RESULTS

3.1. Comparison between FCXM and CDCXM

From a total of 166 deceased donors, 1,312 crossmatches were performed during the year 2010. Sera without PRA evaluation and recipients with missing data were excluded from the analysis. From 1,312 sera samples, 713 crossmatches results were available for all methods: FCXM, CDCXM and SAB from 78 deceased donors. For each donor, sera from a mean of 6.74 ± 3.38 patients were selected for cross matching.

AHGXM results were negative in 537 (75.3%) samples and positive in 176 (24.7). Among positive tests, the following distribution was observed according to the categorical score: 2 = 32 (4.5%); 4 =11 (1.5%), 6 = 18 (2.5%) and 8 = 115 (16.1%). CDCXM for B cells was negative in 486 (68.2%) and positive in 227 (31.8%) crossmatches. Similarly, positive B cells CDCXM were categorized by score: 2=38 (5.3%); 4=27 (3.8%), 6=35 (4.9%) and 8=127 (17.8%). Most of the FCXM for T and B cells were negative (T cells: n = 474; 66.5% and B cells: n = 424; 59.5%).

Furthermore, we analyzed separately the data for T and B cells (Tables 1 and 2 respectively). All positive AHGXM and CDCXM were also FCXM positive (sensitivity = 100%). FCXM specificity was similar for T and B cells (T cells: 88%; B cells: 87%). FCXM accuracy was 91%.

Table 1. Comparison between AHGXM and FCXM tests results for T cells.

T cells - n (%)			
	AHGXM		
	Negative	Positive	
FCXM			
Negative	474 (66.5)	-	Kappa = 0.788
Positive	63 (8.8)	176 (24.7)	P ≤ 0.001

AHG, Antihuman Globulin enhanced complement dependent cytotoxic crossmatch; FCXM, flow cytometry crossmatch.

Table 2. Comparison between CDCXM and FCXM tests results for B cells.

Bcells - n (%)			
	CDCXM		
	Negative	Positive	
FCXM			
Negative	424 (59.5)	-	Kappa = 0.813
Positive	62 (8.7)	227 (31.8)	P ≤ 0.001

CDCXM, complement dependent cytotoxic crossmatch; FCXM, flow cytometry crossmatch.

3.2. Presence of DSA evaluation in CDCXM and FCXM

Three hundred eight seven patients (54.3%) had a class I or II DSA in the total sample and 326 (45.7%) did not present DSAs for HLA-A, -B and –DRB1. Anti-HLA-C, DQB1 and DPB1 were detected in same samples but were not considered DSAs as

HLA-C, HLA-DQ e HLA-DP loci typing were not available. Anti-HLA-C was absent in 452 (63.4%), but DQB1or DPB1 were present in 371 (52%) of the positive PRA class II.

The agreement between CDCXM and presence of DSAs (T cells: Kappa=0.572; B cells: Kappa = 0.627) was not as good as the FCXM and presence of DSAs (T cells: Kappa=0.732; B cells: Kappa=0.735). Data is summarized in tables 3 and 4.

Table 3. Comparison between AHGXM and DSA tests results for T cells.

T cells - n (%)			
	DSA		
	Negative	Positive	
AHGXM			
Negative	409 (57.4)	128 (18.0)	Kappa = 0.572
Positive	10 (1.4)	165 (23.1)	$p \leq 0.001$
FCXM			
Negative	402 (56.4)	72 (10.1)	Kappa = 0.732
Positive	7 (2.4)	221 (31.0)	$p \leq 0.001$

AHG, Antihuman Globulin; CDCXM, complement dependent cytotoxic crossmatch; FCXM, flow cytometry crossmatch; DSA, donor-specific antibodies.

Table 4. Comparison between CDCXM and DSA tests results for B cells.

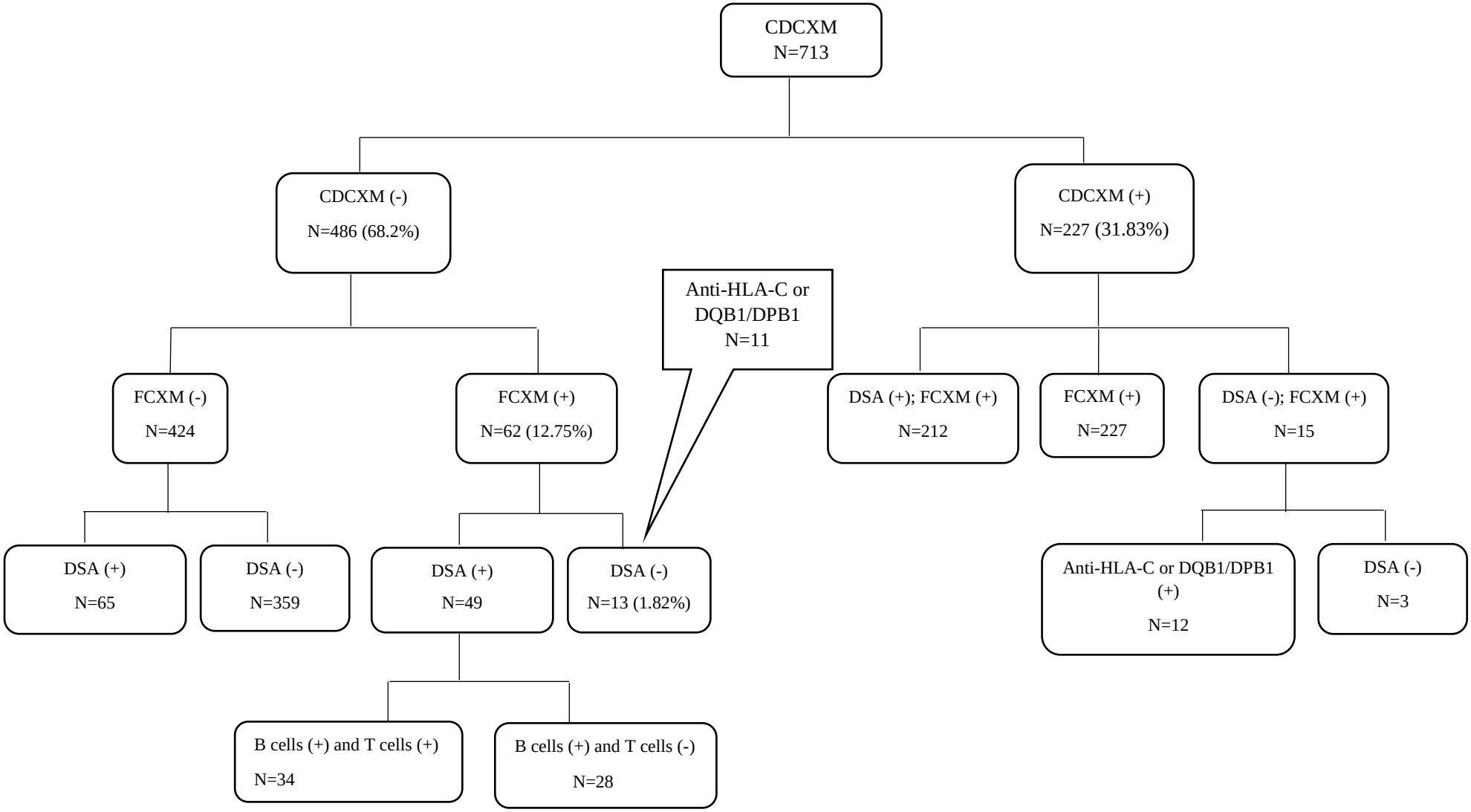
B cells - n (%)			
	DSA		
	Negative	Positive	
CDCXM			
Negative	372 (52.2)	114 (16.0)	Kappa = 0.627
Positive	15 (2.1)	212 (29.7)	$p \leq 0.001$
FCXM			
Negative	359 (50.4)	65 (9.1)	Kappa = 0.735
Positive	28 (3.9)	261 (36.6)	$p \leq 0.001$

CDCXM, complement dependent cytotoxic crossmatch; FCXM, flow cytometry crossmatch; DSA, donor-specific antibodies.

The median sum MFI for anti-HLA class I was 10,130 (IQR= 3,506 – 19,347) and 5,315 (IQR=1,956 – 11,977) for anti-HLA class II.

We analyzed CDCXM/AHGXM and FCXM considering the presence of DSA (Figure 1). Among the CDCXM negative tests (N= 486; 68.17%), 62 (12.75%) were FCXM positive. When the DSA presence was considered only 13 cases (1.82%) remained negative.

Figure 1. Flowchart of the comparison between CDCXM and FCXM considering the presence of DSA.

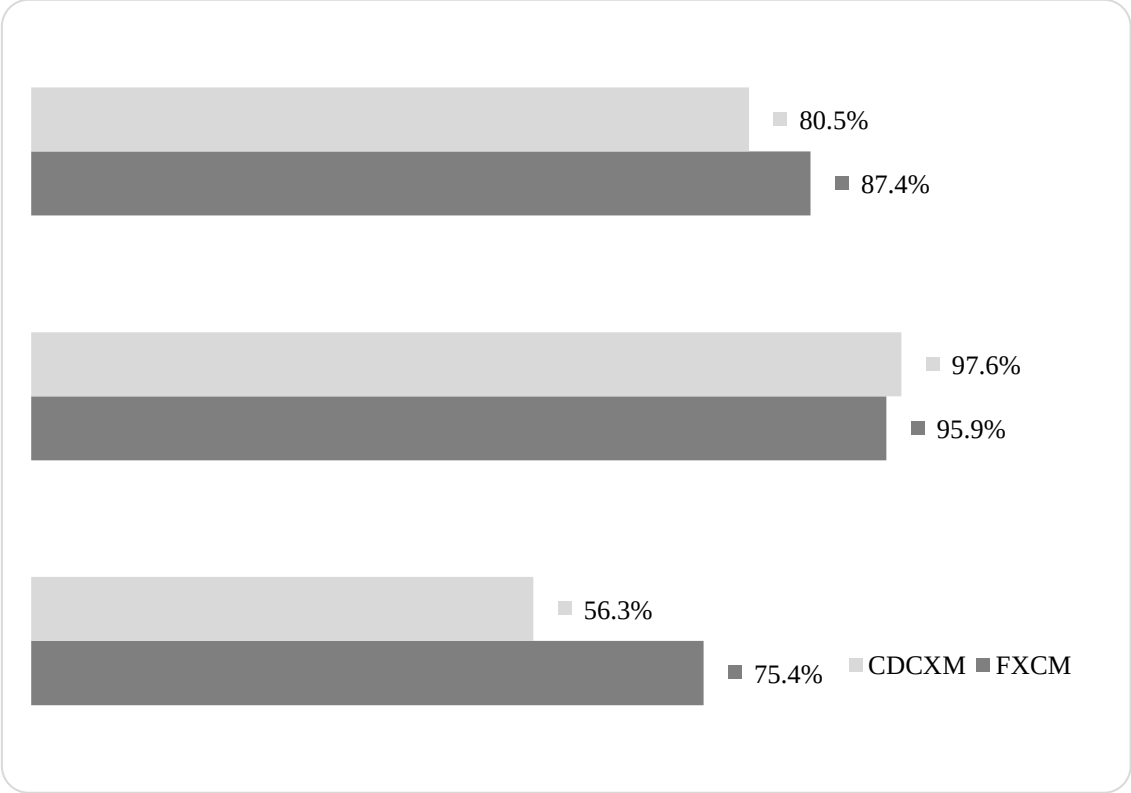


CDCXM, complement-dependent cytotoxicity crossmatch for T and B cells; CDCXM (+), B cells positive with T cells negative or positive; CDCXM (-), negative for T and B cells; FCXM, flow cytometric crossmatch for T and B cells; FCXM (+),B cells positive with T cells negative or positive; FCXM (-),negative for T and B cells; DSA (+), presence of the anti-HLA class I or class II; DSA (-), anti-HLA Known absent.

Considering the presence of DSA as a gold standard, we found T and B cells FCXM to be more accurate (T cells: 87.4% and B cells: 86.9%) than the CDCXM (T cells: 80.5% and B cells: 81.9%). The sensitivity and specificity were show in Figures 2a and 2b.

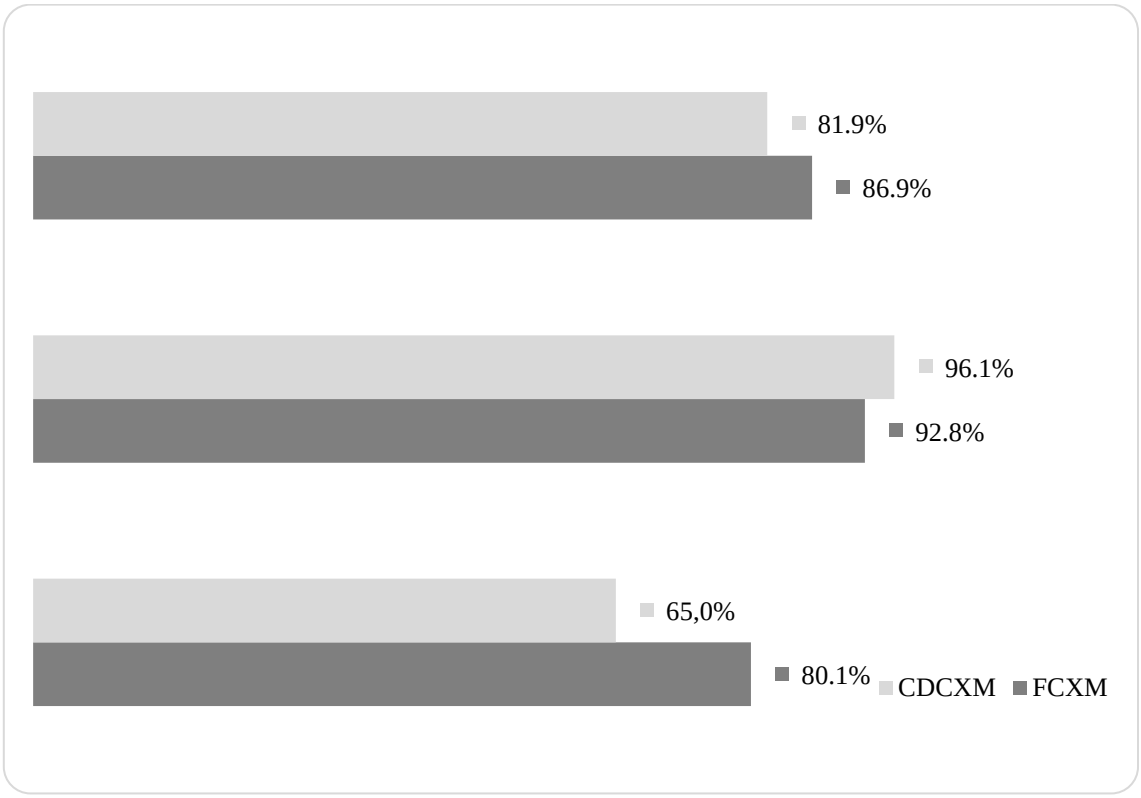
Figure 2: Comparison of CDCXM and FCXM with the presence of DSA.

A. T cells



CDCXM, complement dependent cytotoxic crossmatch; FCXM, flow cytometry crossmatch.

B. B cells



CDCXM, complement dependent cytotoxic crossmatch; FCXM, flow cytometry crossmatch.

4. DISCUSSION

In the present study a good agreement between pre renal transplantation CDCXM and FCXM for T and B cells was found. False positive results in FCXM were 1.82% when DSA results were imputed in the analysis. The sensitivity was higher in T and B cells FCXM as compared with the CDCXM cross match results, with in the same sera and cells. However, as expected, CDCXM demonstrated higher specificity than FCXM. In brief this was a retrospective cross-sectional study, using crossmatch results performed during the year 2010. Three distinct crossmatch methods, using the same cut-off to assess all samples, were performed and all types of donors (standard and expanded criteria) were included. All tests were carried out and analyzed by two biomedical professionals in order to add confidence to the results.

There is a paucity of studies analyzing paired cross match methods in organ transplantation. Ayna*et. al.*[10], in a study including 47 kidney transplant candidates, performed a concordance analysis of the CDCXM and FCXM cross matches and found a concordance percentage of 76.6%. Among their discordant results, 9 (19.1%) were negative CDCXM and positive FCXM. These numbers are in accordance with our data before the imputing of the results of the SAB-DSA analyzes. In another studies it has been shown that previously undetected DSAs were associated with early graft loss due to antibody-mediated rejection, multiple and severe acute rejections, and a higher incidence of subclinical rejection [11,12]. In the present study it was found that 15% of the DSA positive tests resulted CDCXM negative.The impact of DSA positive tests in renal transplant patients was evaluated by Wu *et. al.*who found that 5% of 221 kidney transplant recipients with negative pre-transplant CDCXM experienced antibody mediated rejection [13]. Another recent study analyzed the physical crossmatch tests with presence of DSA and concluded that in crossmatches performed against DSA, the best performance was observed with FCXM (83%) as compared to DSA detection (71%) and CDCXM (66%).[14]. Also, according to Graff *et. al.*[15], positive FCXM has important prognostic implications even when CDCXM is negative and these authors proposed that FCXM should not routinely be dismissed as "overly sensitive" when CDCXM results are negative. With the approach used in the present study it was shown that a low frequency of false positive FCXM is to be expected.

The present study has a few limitations: (a) HLA typing for loci HLA-C, HLA-DQB1 e HLA-DPB1 was not available and hindered DSA assessment for these loci, those cases were identified and analyzed separately to minimize the effects of this limitation; (b) We did not identify which patients underwent kidney transplantation or followed up the graft survival as these were out of the scope of this study.

Summarizing, the main purpose of the present study was to determine the number of FCXM false positive results. When analyzed together with the SAB results, the number of FCXM false positive results was substantially low. The sensitivity of the SAB and FCXM was similar and we observed a better agreement than with CDCXM. However, many factors could be affecting FCXM and SAB assays, a major limitation is inter and intra-laboratory variability in test results [16,17]. We also suggest that virtual crossmatch results should be evaluated together with physical crossmatch results and according to most transplant centers guidelines, the presence of DSA is a contraindication for transplantation [18,19]. Finally, we suggest that the validation of the tests, in each laboratory and transplant center, is imperative.

Acknowledgments

The authors would like to thank the Transplantation Immunology Laboratory team for the effort in performing all HLA assays.

REFERENCES

[1] T.P.I. Patel Ramon, Significance of the positive crossmatch test in kidney transplantation, N. Engl. J. Med. 280 (1969) 735–739.

[2] P.I. Terasaki, A personal perspective: 100-Year history of the humoral theory of transplantation, Transplantation. 93 (2012) 751–756. https://doi.org/10.1097/TP.0b013e3182483713.

[3] H.M. Gebel, R.A. Bray, P. Nickerson, Pre-Transplant Assessment of Donor-Reactive, HLA-Specific Antibodies in Renal Transplantation: Contraindication vs. Risk, Am. J. Transplant. 3 (2003) 1488–1500. https://doi.org/10.1046/j.1600-6135.2003.00273.x.

[4] K. Tinckam, Histocompatibility methods, Transplant. Rev. 23 (2009) 80–93. https://doi.org/10.1016/j.trre.2009.01.001.

[5] Bray RLebeck LGebel H, The flow cytometric crossmatch. Dual-color analysis of T cell and B cell reactivities, Transplatation. 48 (1989) 834–840.

[6] K. Jayant, I. Reccia, B.M. Julie, A. Sharma, A. Halawa, Role of crossmatch testing when Luminex-SAB is negative in renal transplantation, Polish J. Surg. 90 (2018) 42–46. https://doi.org/10.5604/01.3001.0011.5959.

[7] B.D. Tait, C. Süsal, H.M. Gebel, P.W. Nickerson, A.A. Zachary, F.H.J. Claas, E.F. Reed, R.A. Bray, P. Campbell, J.R. Chapman, P.T. Coates, R.B. Colvin, E. Cozzi, I.I.N. Doxiadis, S. V. Fuggle, J. Gill, D. Glotz, N. Lachmann, T. Mohanakumar, N. Suciu-Foca, S. Sumitran-Holgersson, K. Tanabe, C.J. Taylor, D.B. Tyan, A. Webster, A. Zeevi, G. Opelz, Consensus guidelines on the testing and clinical management issues associated with HLA and Non-HLA antibodies in transplantation, Transplantation. 95 (2013) 19–47. https://doi.org/10.1097/TP.0b013e31827a19cc.

[8] A.B. Hahn, G.A. Land, R.M. Strothman, American Society for Histocompatibility: Laboratory Manual, 2000.

[9] R.S. Liwski, H.M. Gebel, Of Cells and Microparticles: Assets and Liabilities of HLA Antibody Detection, Transplantation. 102 (2018) S1–S6. https://doi.org/10.1097/TP.0000000000001818.

[10] T.K. Ayna, M. Soyöz, Y. Kurtulmuş, S.M. Doğan, B. Özyilmaz, C. Tuğmen, I. Pirim, Comparison of complement-dependent cytotoxic and flow-cytometry crossmatch results before cadaveric kidney

transplantation, Transplant. Proc. 45 (2013) 878–880. https://doi.org/10.1016/j.transproceed.2013.02.083.

[11] M. Karpinski, D. Rush, J. Jeffery, M. Exner, H. Regele, S. Dancea, D. Pochinco, P. Birk, P. Nickerson, Flow Cytometric Crossmatching in Primary Renal Transplant Recipients with a Negative Anti-Human Globulin Enhanced Cytotoxicity Crossmatch Materials and Methods Study Population, J Am Soc Nephrol. 12 (2001) 2807–2814.

[12] I. Salvadé, V. Aubert, J.P. Venetz, D. Golshayan, A.C. Saouli, M. Matter, S. Rotman, G. Pantaleo, M. Pascual, Clinically-relevant threshold of preformed donor-specific anti-HLA antibodies in kidney transplantation, Hum. Immunol. 77 (2016) 483–489. https://doi.org/10.1016/j.humimm.2016.04.010.

[13] P. Wu, J. Jin, M.J. Everly, C. Lin, P.I. Terasaki, J. Chen, Impact of alloantibody strength in crossmatch negative DSA positive kidney transplantation, Clin. Biochem. 46 (2013) 1389–1393. https://doi.org/10.1016/j.clinbiochem.2013.05.053.

[14] J.P. Peräsaari, T. Jaatinen, J. Merenmies, Donor-specific HLA antibodies in predicting crossmatch outcome: Comparison of three different laboratory techniques, Transpl. Immunol. 46 (2017) 23–28. https://doi.org/10.1016/j.trim.2017.11.002.

[15] R.J. Graff, P.M. Buchanan, N. Dzebisashvili, M.A. Schnitzler, J. Tuttle-Newhall, H. Xiao, E. Schadde, A. Gheorghian, K.L. Lentine, The clinical importance of flow cytometry crossmatch in the context of CDC crossmatch results, Transplant. Proc. 42 (2010) 3471–3474. https://doi.org/10.1016/j.transproceed.2010.06.025.

[16] R.S. Liwski, A.L. Greenshields, R.A. Bray, H.M. Gebel, Becoming a chef in the human leukocyte antigen kitchen: Interpretation and modification of human leukocyte antigen-antibody assays, Curr. Opin. Organ Transplant. 22 (2017) 407–414. https://doi.org/10.1097/MOT.0000000000000423.

[17] A. Konvalinka, K. Tinckam, Utility of HLA Antibody Testing in Kidney Transplantation, J. Am. Soc. Nephrol. 26 (2015) 1489–1502. https://doi.org/10.1681/ASN.2014080837.

[18] G. Schlaf, B. Pollok-Kopp, E. Schabel, W. Altermann, Case Report Artificially Positive Crossmatches Not Leading to the Refusal of Kidney Donations due to the Usage of Adequate Diagnostic Tools, Case Rep. Transplant. 2013 (2013) 1–7. https://doi.org/10.1155/2013/746395.

[19] D.L. Roelen, I.I.N. Doxiadis, F.H.J. Claas, Detection and clinical relevance of donor specific HLA

antibodies: A matter of debate, Transpl. Int. 25 (2012) 604–610. https://doi.org/10.1111/j.1432-2277.2012.01491.x.

8. CONSIDERAÇÕES FINAIS

O objetivo principal deste estudo foi avaliar o impacto laboratorial e clínico de novos protocolos utilizados na avaliação imunológica pré-transplante renal. A partir dos resultados obtidos, entendemos que a tomada de decisão clínica com uma prova cruzada real mais sensível, possibilita a segurança de transplantar pacientes na presença de anticorpos anti-HLA do doador frente a uma prova cruzada negativa pois o ensaio de fase sólida, *single antigen beads* possui limitações, entre elas um alto coeficiente de variação intra e inter-laboratorial, bem como a padronização das análises[26].

No estudo diagnóstico retrospectivo do ano de 2010, o protocolo de prova cruzada CFXM utilizado demonstrou uma acurácia de 91% quando comparamos com o CDCXM que foi o padrão-ouro utilizado. Como esperado, por se tratar de uma metodologia mais sensível onde são detectados anticorpos fixadores e não fixadores de complemento, a sensibilidade da CFXM foi de 100% e a especificidade foi de 88% para linfócitos T e 87% para linfócitos B. A correlação dos resultados foi estatisticamente significativa tanto para linfócitos T (kappa = 0,788; p = <0,001) quanto para linfócitos B (kappa = 0,813; p = <0,001). Por ser um teste com alta sensibilidade a CFXM tem sido questionada em relação aos resultados falsos positivos. O presente estudo quantificou os casos em que pacientes com resultados CDCXM negativos tiveram correspondentes resultados positivos na CFXM. Dos 486 CDCXM negativos, 62 (12,75%) foram positivos na CFXM. Analisamos estes casos quanto a VXM, e 49 pacientes apresentaram AAD, destes, 11 pacientes tinham PRA positivo (com anticorpos anti-HLA-C, HLA-DQB1 e HLA-DPB1 doadores sem tipagem para esses loci) e 2 PRA negativos. Considerando que independentemente do resultado da CFXM um resultado de CDCXM negativo com a presença de AAD aumentaria o risco de ocorrência de rejeição aguda humoral, uma análise de AAD mais aprofundada seria recomendada. Logo, encontramos em nossa amostra 1,82% de resultados falsos positivos de acordo com as considerações acima. A partir destas análises concluímos que, o laboratório de histocompatibilidade deve estar alinhado às diretrizes clínicas dos grupos transplantadores, considerando o perfil de sensibilização imunológica prévia dos pacientes.

Em relação a validação clínica do CFXM protocolo HALIFAX, em um ano de acompanhamento do grupo de pacientes transplantados com CFXM (teste) e de um grupo de pacientes transplantados com CDCXM (controle) não houve diferença na sobrevida entre os grupos tanto do paciente (p = 0,591) quanto do enxerto (p = 0,692). Além disso, não houve diferença na incidência de rejeição celular aguda ≥ 1A entre os grupos. Acreditamos que este resultado valida clinicamente o uso da CFXM como teste único pré transplante. Em acordo com a maior sensibilidade do CFXM não encontramos episódios de RHA na coorte avaliada por esse teste, enquanto que na coorte controle houveram 3 casos de RHA. Ao contrário da nossa expectativa, não obtivemos impacto significativo no tempo de isquemia fria (p = 0,342), esperávamos uma diminuição neste fator preditor de risco por conta do protocolo HALIFAX possuir tempos menores de incubação e centrifugação.

9. PERSPECTIVAS FUTURAS

Investigações e análises subsequentes com as coortes estudadas poderiam ser realizadas e incluem:

9.1. Avaliar os desfechos clínicos dos pacientes transplantados com CDCXM negativo e CFXM positivo, estratificando risco de acordo com o desvio de canal;

9.2. Prolongar o tempo de acompanhamento das coortes CDCXM e CFXM e avaliar os desfechos clínicos;

9.3. Avaliar o impacto do tempo de liberação da prova cruzada nos tempos de isquemia fria do órgão.

10. ANEXOS

10.1- *Checklist*STROBE

O *checklist* STROBE foi utilizado para elaboração deste trabalho e as páginas referem-se ao manuscrito 7.3.1.

Section and Topic	Item No	Recommendation	On page #
Titleand abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	27
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	27
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	29
Objectives	3	State specific objectives, including any prespecified hypotheses	29
Methods			
Study design	4	Present key elements of study design early in the paper	30
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	30
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describemethodsof follow-up	30
		(*b*)For matched studies, give matching criteria and number of exposed and unexposed	NA
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Givediagnosticcriteria, ifapplicable	32
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	30-32
Bias	9	Describe any efforts to address potential sources of bias	30-32
Studysize	10	Explain how the study size was arrived at	30
Quantitativevariables	11	Explain how quantitative variables were handled in the analyses. Ifapplicable, describewhichgroupingswerechosenandwhy	30-32
Statisticalmethods	12	(*a*) Describe all statistical methods, including those used to control for confounding	32
		(*b*) Describe any methods used to examine subgroups and interactions	32
		(*c*) Explain how missing data were addressed	32
		(*d*) If applicable, explain how loss to follow-up was addressed	32
		(*e*) Describe any sensitivity analyses	32
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	33
		(b) Give reasons for non-participation at each stage	33
		(c) Consider use of a flow diagram	NA
Descriptivedata	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	34-35
		(b) Indicate number of participants with missing data for each variable of interest	33-35
		(c) Summarise follow-up time (eg, average and total amount)	39

Outcome data	15*	Report numbers of outcome events or summary measures over time	39-40
Mainresults	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	NA
		(*b*) Report category boundaries when continuous variables were categorized	33
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	NA
Otheranalyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	NA
Discussion			
Key results	18	Summarise key results with reference to study objectives	41
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discussbothdirectionand magnitude ofanypotential bias	43
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	41-43
Generalisability	21	Discuss the generalisability (external validity) of the study results	41
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	NA

10.2- *Checklist*STARD

O *checklist* STARD foi utilizado para elaboração deste trabalho e as páginas referem-se ao manuscrito 7.3.2.

Section and Topic	Item #	Recommendation	On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	50
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	53
METHODS	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	53-54
Participants	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	53
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	54
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	54
Test methods	7	The reference standard and its rationale.	54
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	53-55
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	53-55
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	55
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	NA
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	55
	13	Methods for calculating test reproducibility, if done.	55
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	56
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	NA
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	56
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	NA
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	NA
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	NA
	20	Any adverse events from performing the index tests or the reference standard.	NA
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	56
	22	How indeterminate results, missing data and outliers of the index tests were handled.	NA
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	NA
	24	Estimates of test reproducibility, if done.	NA
DISCUSSION	25	Discuss the clinical applicability of the study findings.	62