UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS:

ENDOCRINOLOGIA

ESTUDO DA ASSOCIAÇÃO ENTRE O POLIMORFISMO K121Q NO GENE *E-NPP1* E DOENÇA RENAL DO DIABETES E REJEIÇÃO AGUDA EM TRANSPLANTADOS RENAIS

TESE DE DOUTORADO

DENISE ALVES SORTICA

PORTO ALEGRE, AGOSTO DE 2017.

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TESE DE DOUTORADO APRESENTADA AO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA DA UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL (UFRGS) COMO REQUISITO PARCIAL PARA OBTENÇÃO DO TÍTULO DE DOUTOR EM ENDOCRINOLOGIA.

PORTO ALEGRE, AGOSTO DE 2017.

S714e Sortica, Denise Alves

Estudo do polimorfismo K121Q no gene ENPP1 na doença renal do diabetes e na rejeição aguda do transplante renal / Denise Alves Sortica. – 2017.

78 f.

Orientador: Luis Henrique Canani, Deise Crispim Moreira

Coorientador: Roberto Ceratti Manfro

Tese (Doutorado) – Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Ciências Médicas: Epidemiologia, Porto Alegre-RS, 2017.

1. Diabetes mellitus. 2. Nefropatias. 3. Polimorfismo genético. I. Canani, Roberto Ceratti, orient. II. Moreira, Deise Crispim, orient. III. Manfro, Roberto Ceratti, coorient. IV. Título

Dados Internacionais de Catalogação na Publicação (CIP)
Biblioteca FAMED/HCPA

DEDICO A MINHA FAMÍLIA

“Não sabendo que era impossível, foi lá e fez”.

Jean Cocteau

AGRADECIMENTOS

Prof. Canani, o meu Professor Robin Hood, que da maneira mais insólita, me deu a vara de pescar e a partir disso a oportunidade de conhecer um mundo que eu nem imaginava existir, o mundo da pesquisa, mudando o rumo da minha vida para sempre. Minha vida se resume: antes de LHC e depois de LHC. Gratidão eterna.

Prof[a]. Daisy Crispim, que além de orientadora, considero minha irmã, que me ensinou absolutamente tudo na pesquisa, com uma paciência de um monge tibetano e nunca desistiu de mim, e sem a sua orientação, essa tese de maneira alguma existiria, dizer obrigada é muito pouco, uma palavra adequada ainda não inventaram, portanto tens minha gratidão infinita.

Prof[a]. Andrea Bauer, que sem sua colaboração incansável e dedicação de inúmeras horas para este trabalho, a execução do mesmo não seria possível.

As minhas amigas, Prof[a]. Cristiane Leitão e Dr[a]. Fabiana Valiatti pelas considerações sempre pertinentes e apoio completamente altruísta.

Prof. Manfro pela co-orientação e o Serviço se Nefrologia pelo espaço concedido.

Bruna Nicolletto, que me deu suporte técnico para o trabalho, mas principalmente emocional, me ajudou a acreditar que daria tudo certo, e tornou minha vida mais feliz, obrigada por existir, e me dar apoio.

A Bianca Marmontel e Ana Paula Bouças por existirem e acrescentarem a minha vida o conhecimento de uma amizade verdadeira e paciência.

Pâmela, minha pupila, sempre ao meu lado, de forma silenciosa e outras nem tanto, mas não concebo mais viver sem sua presença, apoio e colaboração.

Aos alunos de iniciação cientifica Guilherme Zaffari, Andressa Santer, Laura Olivo, Riciéli Crestani e Jennifer Staehler, meus braços direito e esquerdo, colaboradores incansáveis, agradeço de coração.

Aos meus colegas e amigos de laboratório: Letícia Brondani, Taís Assmann, Marjorie Buffon, Natalia Emerim, Guilherme Coutinho, Jakeline Rheinheimer, Joana Lemos, Carla Krause, Carla Vaz, Lucieli Ceolin, Poliana Espindola e Simone Wajner.

Minha família biológica e não biológica por existirem na minha vida: Antônio Sortica, Nilza Alves Sortica, Ana Helena Alves Sortica, Paulo Roberto Aymone Filho, Amanda Brito de Freitas e Marisa Teushel.

Lucas Mendes, médico e amigo, por não me deixar enlouquecer e me dar força pra viver.

Esta tese de doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de uma breve introdução geral sobre o assunto e dois manuscritos sobre o tema da tese, seguidos de perspectivas.

-**Artigo original 1**: "Association between the *E-NPP1* K121Q polymorphism and risk of diabetic kidney disease: a systematic review and meta-analysis." (Publicado na revista *Plos One* em 2015).

-**Artigo original 2**: "*Ectonucleotide Pyrophosphatase/Phosphodiesterase 1* K121Q polymorphism is associated with acute kidney rejection." (A ser submetido à revista *Clinical Journal of the American Society of Nephrology*).

LISTA DE ABREVIATURAS PARA A INTRODUÇÃO

ABTO	Associação Brasileira de Transplante de Órgãos
DM	Diabetes Mellitus
DM1	Diabetes mellitus Tipo 1
DM2	Diabetes mellitus Tipo 2
DRCT	Doença renal crônica terminal
DRD	Doença renal do diabetes
E-NPP1	*Ectonucleotide pyrophosphatase/phosphodiesterase*
EUA	Excreção urinária de albumina
HAS	Hipertensão arterial sistêmica
HLA	Antígeno leucocitário humano
HOMA-IR	*Homeostasis model assessment – insulin resistance*
HR	*Harzard ratio*
IDF	*International Diabetes Federation*
IRS1	*Insulin receptor substrate 1*
KDIGO	*Kidney Disease Improving Global Outcomes*
ENTPDase	*Ectonucleosideo trifosfato difosfohidrolase*
NKF	*National Kidney Foundation*
PC-1	*Plasma cell differentiation antigen-1*
RA	Rejeição aguda
RC	Razão de chance
TFG	Taxa de filtração glomerular
UNOS	*United Network for Organ Sharing*

LISTA DE ABREVIATURAS PARA OS ARTIGOS ORIGINAIS

ACR	Albumin Creatinine Ratio
AR	Acute rejection
ATPase	Adenosine triphosphatase
BMI	Body mass index
CI	Confidence intervals
DGF	Delayed graft function
DKD	Diabetic kidney disease
DM	Diabetes mellitus
E-NPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
EASD	European Association for the Study of Diabetes
ESRD	End-stage renal disease
FEM	Fixed effect model
GFR	Glomerular filtration rate
HR	Hazard Ratio
HWE	Hardy-Weinberg equilibrium
IR	Insulin resistance
MeSH	Medical subject headings
MOOSE	Meta-analysis of Observational Studies in Epidemiology
NOS	Newcastle-Ottawa Scale
NPPs	Ectonucleotide pyrophosphatase/phosphodiesterases
NTPDases	Ectonucleoside triphosphate diphosphohydrolases
OR	Odds ratios

PC-1	Plasma-cell differentiation antigen-1
PCR-SSO	Polymerase chain reaction – sequence specific oligonucleotide
PCR-SSP	Polymerase chain reaction – sequence specific primers
Pi	Inorganic phosphate
PPi	Pyrophosphate
PRA	Panel reactive antibody
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
T2DM	Type 2 diabetes mellitus
Treg	T regulatory cells
UAE	Urinary albumin excretion

SUMÁRIO

RESUMO

A E-NPP1 (*ecto-nucleotide pyrophosphatase* / *phosphodiesterase* 1) faz parte de uma família de enzimas responsáveis por hidrolisar nucleotídeos extracelulares, conhecidas como ectonucleotidases. Esta enzima foi inicialmente identificada como um marcador de superfície de linfócitos B recebendo o nome de *plasma-cell differentiation antigen-1* (PC-1). O efeito do aumento da expressão de *E-NPP1* foi descrito inicialmente em uma paciente com uma rara resistência extrema à insulina. Nessa paciente, a inibição marcada da função do receptor da insulina foi devido ao aumento da expressão de *E-NPP1*. Estudos subsequentes realizados em células humanas confirmaram esse achado.

De acordo com o papel da E-NPP1 na resistência à insulina, diversos estudos posteriores demonstraram a associação de polimorfismos no gene *E-NPP1* com diabetes mellitus (DM), o que parece ser dependente do grupo étnico analisado. O polimorfismo K121Q (rs1044498) é de longe o mais estudado neste gene e causa a troca de uma lisina (K) para glutamina (Q) no códon 121 (éxon 4). O polimorfismo K121Q parece interferir com interações proteína-proteína uma vez que ele ocorre no segundo domínio *somatomedin-B-like* da E-NPP1, o qual é importante para essa função. De fato, estudos *in vitro* demonstraram que o alelo Q interage mais fortemente com o receptor da insulina do que o alelo K, reduzindo a autofosforilação deste receptor. O alelo Q também reduz a fosforilação do substrato do receptor da insulina 1 (*insulin receptor substrate 1* – IRS1) induzida por insulina, a atividade de quinase do fosfatidilinositol-3, a síntese de glicogênio e a proliferação celular. Além disso, um estudo demonstrou que sicilianos portadores do alelo Q tiveram menor sensibilidade à insulina do que

indivíduos não-portadores. Resultados similares foram obtidos em estudos realizados em finlandeses e suecos, mas não em dinamarqueses.

O polimorfismo K121Q também parece estar associado com doença renal do diabetes (DRD), embora os estudos ainda sejam contraditórios. Diante disso, realizamos uma revisão sistemática e metanálise dos estudos disponíveis na literatura visando avaliar se o polimorfismo K121Q no gene *E-NPP1* está realmente associado com a DRD. A metanálise incluiu 7 estudos observacionais elegíveis, totalizando 3571 indivíduos analisados [1606 com DRD (casos) e 1965 sem DRD (controles)]. A análise quantitativa destes estudos demonstrou a associação significativa entre o genótipo Q/Q do polimorfismo K121Q e risco para DRD nas populações europeia [razão de chances (RC) = 1,79, IC 95% 1,22 – 2,76] e asiática (RC = 2,15, IC 95% 1,23 – 3,75).

Interessantemente, alguns estudos demonstraram que alguns membros da família das ectonucleotidases estão associados com dano renal, imunidade e rejeição crônica. Entretanto, até o momento, a E-NPP1, mesmo estando associada com DRD, ainda não foi avaliada no contexto da rejeição aguda (RA) em pacientes transplantados. Dessa forma, também tivemos como objetivo avaliar a associação do polimorfismo K121Q no gene *E-NPP1* com RA em pacientes transplantados renais. Em um estudo de caso-controle, o polimorfismo K121Q foi genotipado em 454 pacientes transplantados renais do sul do Brasil, sendo 96 pacientes com RA (casos) e 358 sem RA (controles).

A frequência do alelo Q foi maior no grupo de indivíduos com RA do que no grupo sem RA (24,5% *vs*. 17,0%, p = 0,024). Após controle por possíveis fatores de confusão, o genótipo Q/Q permaneceu como um preditor independente para RA comparado a portadores do alelo K [*Harzard ratio* (HR) = 2,76, IC 95% 1,44 – 5,30].

Em conclusão, nosso estudo de revisão sistemática e metanálise confirmou a associação do genótipo Q/Q do polimorfismo K121Q no gene *E-NPP1* com risco para DRD. Além disso, nosso estudo em pacientes transplantados renais demonstrou, pioneiramente, a associação do genótipo Q/Q deste polimorfismo com risco para RA. Estudos adicionais são necessários para replicar este achado em outras populações, bem como identificar o mecanismo molecular explicando esta associação.

ABSTRACT

E-NPP1(ectonucleotide pirophosphatase/phosphodiesterase 1) belongs to a family of enzymes responsible for hydrolyzing extracellular nucleotides, known as ectonucleotidases. This enzyme was initially identified as a surface marker of B-lymphocytes, called plasma-cell differentiation antigen-1 (PC-1). The effect of E-NPP1 upregulation was initially described in a patient with a rare severe insulin resistance. In this patient, a marked inhibition of the insulin receptor function occurred due to an increase in *E-NPP1* expression. Further studies in human cells have confirmed this finding.

According to the role of E-NPP1 in insulin resistance, several studies have demonstrated the association of *E-NPP1* gene polymorphisms with diabetes mellitus (DM), which seems to be dependent on the ethnic group analyzed. The K121Q polymorphism (rs1044498) is by far the most studied variant in this gene, and exchanges a lysine (K) by a glutamine (Q) in codon 121 (exon 4). The K121Q polymorphism seems to interfere in protein-protein interactions since it occurs in the second domain somatomedin-B-like of the E-NPP1, which is important for that function. In fact, *in vitro* studies demonstrated that the Q allele interacts more strongly with the insulin receptor than the K allele; thus, reducing the autophosphorylation of this receptor. The Q allele also reduces the insulin-induced phosphorylation of the insulin receptor substrate 1 (IRS1), the kinase activity of fosfatidilinositol-3, glycogen synthesis, and cellular proliferation. Moreover, a study demonstrated that Sicilians who carried the Q allele had less sensibility to insulin than non-carriers. Similar results were also obtained in studies performed in Finish and Swedish, but not in Danish people.

The K121Q polymorphism also seems to be associated with diabetic kidney disease (DKD), although studies are still controversial. Therefore, we performed a systematic review and meta-analysis of the studies available in literature to evaluate if the *E-NPP1* K121Q polymorphism is indeed associated with DKD. Meta-analysis included 7 observational studies, totalizing 3571 individuals analyzed [1606 with DKD (cases) and 1965 without DKD (controls)]. The quantitative analysis of the studies demonstrated a significant association between the Q/Q genotype of the K121Q polymorphism and risk for DKD in European [odds ratio (OR) = 1.79, 95% CI 1.22 – 2.76)] and Asian (OR = 2.15, 95% CI 1.23 – 3.75) populations.

Interestingly, some studies demonstrated that other members of the Ectonucleotidase family are associated with kidney damage, immunity and chronic rejection. Even though the E-NPP1 has being associated with DKD, so far, it has not been evaluated in the context of acute rejection (AR) in transplant patients. Thus, we also aimed to evaluate the association between the K121Q polymorphism in the *E-NPP1* gene and AR in kidney transplant patients. In a case-control study, the K121Q polymorphism was genotyped in 454 kidney transplant patients from South of Brazil, being 96 patients with AR (cases) and 358 without AR (controls).

The Q allele frequency was higher in individuals with AR compared to those without AR (24.5% *vs*. 17.0%, P = 0.024). After adjustment for possible co-variables, the Q/Q genotype remained as an independent predictor of AR compared to K allele carriers [Hazard ratio (HR) = 2.76, 95% CI 1.44 – 5.30].

In conclusion, our systematic review and meta-analysis confirmed the association between the Q/Q genotype of the *E-NPP1* K121Q polymorphism and risk for DKD. Moreover, our study in kidney transplant patients demostrated, for the first

time, the association between the Q/Q genotype of this polymorphism and risk for AR. Additional studies are needed to replicate this finding in other populations as well as to identify the molecular mechanism behind this association.

1 INTRODUÇÃO

ECTONUCLEOTIDASES

A E-NPP1 (*ecto-nucleotide pyrophosphatase* / *phosphodiesterase* 1) faz parte de uma família de enzimas responsáveis por hidrolisar nucleotídeos extracelulares, conhecidas como ectonucleotidases. As ectonucleotidases incluem membros das sub-famílias E-NTPDase (ecto-nucleosídeo trifosfato difosfohidrolase*)*, E-NPP (ecto-nucleotídeo pirofosfatase fosfodiesterase), fosfatases alcalinas e ecto-5'-nucleotidase, as quais apresentam ampla distribuição tecidual (1). As ectonucleotidases, além de compor o sistema purinérgico e controlar os níveis de nucleotídeos e nucleosídeos extracelularmente, apresentam diferentes características funcionais e estruturais (2). Essas enzimas podem estar localizadas acopladas na membrana plasmática ou solubilizadas no sangue (3).

As NTPDases são consideradas extremamente eficazes no controle da biodisponibilidade extracelular de ATP e ADP, hidrolisando em diferentes proporções nucleotídeos tri- e difosfatados. A NTPDase 1 (ou CD39) foi primeiramente reconhecida como marcadora de linfócitos B e, entre todas, é a mais investigada, hidrolisando ATP e ADP numa proporção de 1:1. Esta proteína é expressa em linfócitos, plaquetas, endotélio, neurônios, astrócitos, músculo liso e também na forma solúvel ou em microvesículas presentes na corrente sanguínea (4, 5).

A NTPDase 1/CD39 possui um papel relevante na homeostase, controlando níveis de ATP e ADP circulantes e, dessa forma, influenciando processos inflamatórios, formação de trombos e tônus vascular (6). A atividade enzimática e a expressão da NTPDase 1/CD39 é alterada em condições patológicas como cardiopatias, hipertensão

arterial sistêmica (HAS), diabetes mellitus (DM) e câncer, sugerindo uma possível participação desse sistema no desenvolvimento fisiopatológico dessas doenças (7, 8).

As E-NPPs possuem 7 formas diferentes (E-NPP 1-7), porém somente as formas E-NPP1, 2 e 3 apresentam capacidade de hidrolisar nucleotídeos (3). As E-NPP 1 e 3 estão ancoradas à membrana plasmática e podem ser clivadas e liberadas na sua forma solúvel, enquanto que a E-NPP2 atua perifericamente quando secretada para o meio extracelular (1). Os substratos em que as E-NPPs podem atuar não ficam limitados a nucleotídeos, podendo hidrolisar ligações pirofosfato ou fosfodiester. Considerando sua atividade enzimática mais ampla, as E-NPPs 1-3 podem hidrolisar ATP e ADP formando AMP e uma molécula de pirofosfato inorgânico (PPi) (1, 4). A distribuição tecidual das E-NPP 1-3 é bastante diversificada e elas podem ser co-expressas em uma mesma célula. A presença dessas enzimas já foi descrita em diversos tecidos e células, tais como: epitelial, pulmonar, hepático, intestinal e renal (4, 5).

A E-NPP1 é de longe a E-NPP melhor caracterizada, que foi originalmente descoberta na superfície de linfócitos B de camundongos como um antígeno de diferenciação celular plasmático, recebendo o nome de *plasma cell differentiation antigen-1* (PC-1) (9). Diversos estudos relacionaram a E-NPP1 com a sinalização da insulina e a patogênese da resistência à insulina (10).

Muitas vezes se torna difícil à distinção entre os membros das famílias das E-NPPs e E-NTPDases devido sua co-expressão tecidual e por possuírem similaridades nas suas especificidades por substratos. O que se sabe é que muitas vezes estas enzimas trabalham em conjunto ou consecutivamente (11-13).

A ecto- 5’-nucleotidase é outro membro da família das ectonucleotidases, a qual possui 7 subtipos: 5 localizam-se no citosol, uma na matriz mitocondrial e uma é

acoplada na membrana plasmática (denominada ecto-5'-nucleotidase/CD73). Essa última enzima hidrolisa nucleotídeos monofosfatados, incluindo o AMP, e sua principal função é a formação da adenosina no meio extracelular (3, 5). Visto que a adenosina é uma importante molécula sinalizadora de processos fisiológicos e patológicos, a ecto-5'-nucleotidase tem, consequentemente, um papel chave na regulação da filtração tubologlomerular nos rins, formação de trombos e inflamação (14, 15). Além disso, assim como outras ecto-enzimas, a ecto-5'-nucleotidase possui algumas funções não enzimáticas, tais como marcadora de ativação de linfócitos T e adesão celular (16).

A ASSOCIAÇÃO DA E-NPP1 COM O DIABETES MELLITUS E A DOENÇA RENAL DO DIABETES

A E-NPP1 é uma proteína de membrana celular contendo um sítio ativo extracelular que catalisa a liberação do nucleosídeo-5-fosfatase dos nucleotídeos e dos seus derivados. Essa proteína consiste de um domínio intracelular NH2 terminal curto, de um domínio transmembrana único, dois domínios *somatomedin-B-like* e de um domínio *COOH-terminal nuclease like.* A E-NPP1 é um homodímero de 230-260 kDa e sua forma reduzida tem um tamanho molecular de 115-135 kDa, dependendo do tipo celular. A E-NPP1 humana tem 873 aminoácidos e é expressa em vários tecidos, incluindo músculo esquelético, tecido adiposo e rins (17).

Estudos *in vitro* mostraram que a superexpressão de *E-NPP1* inibe a atividade tirosina quinase do receptor da insulina em várias células (18-20), causando resistência à insulina (18, 21, 22). Isso ocorre porque a E-NPP1 parece inibir a transdução de sinal da insulina através da sua interação direta com a subunidade alfa do receptor da insulina (19) (**Figura 1**).

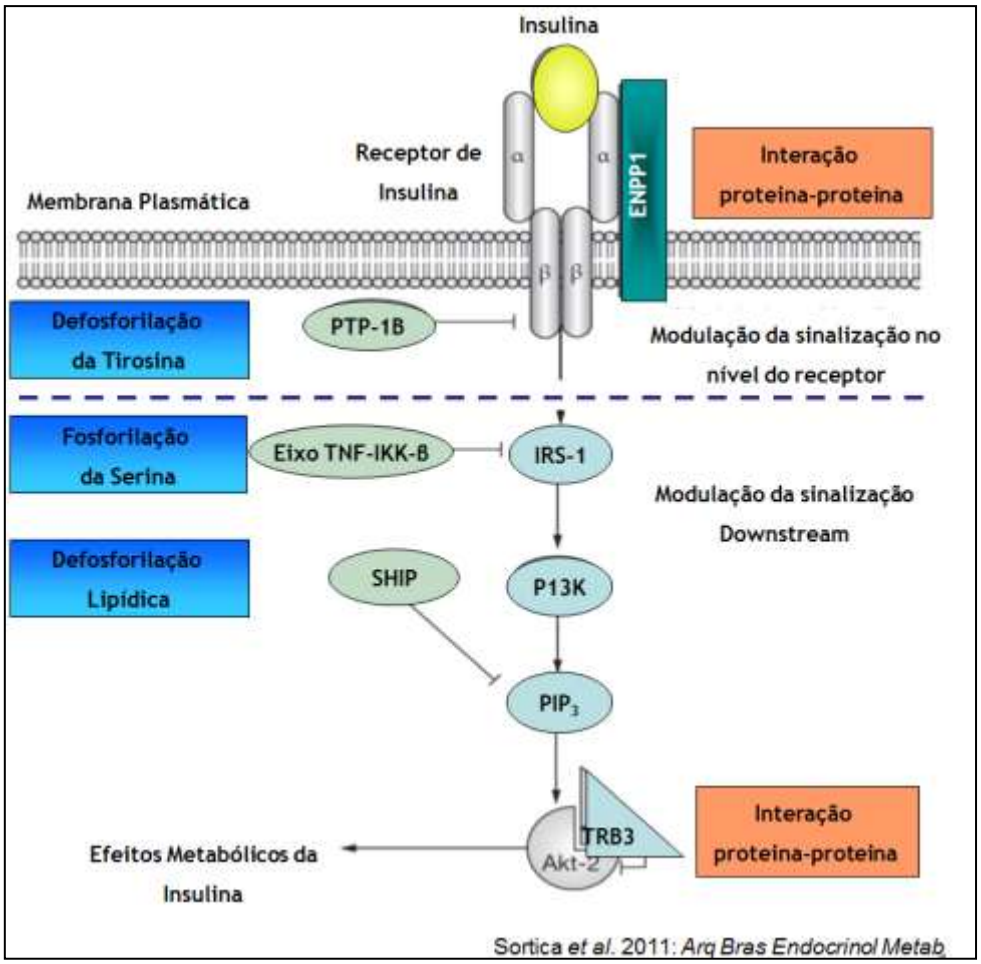

Figura 1. Representação esquemática da cascata de sinalização de insulina [adaptado de Sortica *et al*. (23)].

O gene que codifica a E-NPP1 tem 25 éxons e está localizado no cromossomo 6q22-23 (24) (**Figura 2**). Pizzuti *et al*. (24) descreveram um polimorfismo no éxon 4 deste gene, o qual causa a troca do aminoácido lisina (K) para glutamina (Q) no códon

121 (K121Q; rs1044498). Essa troca de aminoácido está localizada no segundo domínio *somatomedin-B-like* da E-NPP1 e parece interferir com as interações proteína-proteína, uma vez que esse domínio atua nessa função (25). Estudos *in vitro* demonstraram que o alelo Q do polimorfismo K121Q no gene *E-NPP1* interage mais fortemente com o receptor da insulina do que o alelo K, reduzindo a autofosforilação deste receptor (24, 26). O alelo Q também reduz a fosforilação do substrato do receptor da insulina 1 (*insulin receptor substrate 1* – IRS1) induzida por insulina, a atividade de quinase do fosfatidilinositol-3, a síntese de glicogênio e a proliferação celular (26). Além disso, um estudo realizado em uma população siciliana demonstrou que portadores do alelo Q tiveram menor sensibilidade à insulina do que os não-portadores (24). Resultados similares foram obtidos em finlandeses e suecos (27), mas não em dinamarqueses (28).

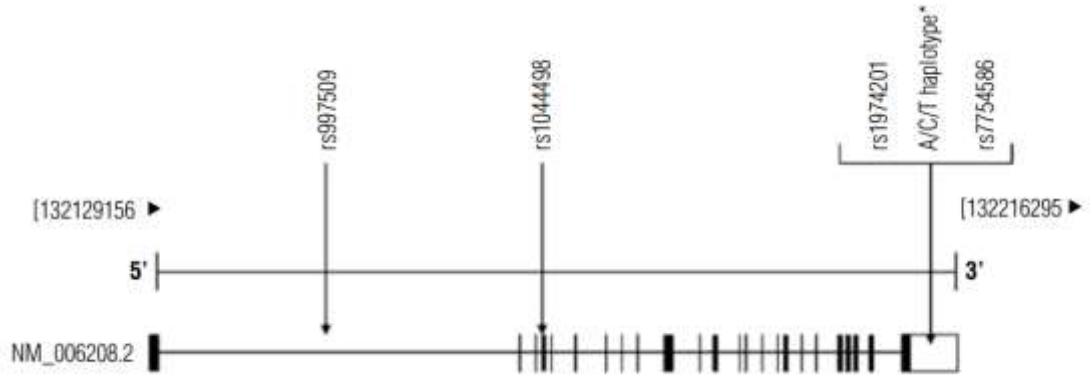

Figura 2. Representação do gene *E-NPP1* no cromossomo 6q22-23 mostrando os principais polimorfismos estudados neste gene, incluindo o K121Q (rs1044498). Adaptado de Sortica *et al*. (23).

De acordo com o envolvimento do polimorfismo K121Q no gene *E-NPP1* com a patogênese da resistência à insulina, este polimorfismo também se mostrou associado com DM em diferentes populações [revisado em (23)]. Posteriormente, esse

polimorfismo também foi estudado em relação a sua associação com a doença renal do diabetes (DRD), embora os resultados dos estudos ainda sejam controversos.

A DRD, até recentemente conhecida como nefropatia diabética, é uma das principais complicações microvasculares do DM, acometendo cerca de 30-50% dos pacientes diabéticos tipo 1 ou tipo 2 (29, 30). Essa complicação é a causa mais comum de doença renal crônica terminal (DRCT) em diversos países e está associada com altas taxas de morbidade e mortalidade entre os pacientes diabéticos (23, 30). Diversos estudos epidemiológicos e de famílias demonstram que fatores ambientais, tais como hiperglicemia, hipertensão arterial e dislipidemia, tem um papel importante no desenvolvimento da DRD em indivíduos geneticamente suscetíveis (30-33). Dessa forma, grandes esforços têm sido focados na identificação de polimorfismos genéticos que sejam associados à DRD.

Na **Tabela 1** encontram-se resumidos os principais resultados dos estudos que avaliaram a associação do polimorfismo K121Q no gene *E-NPP1* em relação à DRD. De Cosmo *et al*. (34) foram os primeiros pesquisadores a descrever que o polimorfismo K121Q está associado à taxa de perda de função renal em pacientes com DM tipo 1 (DM1) e proteinúria. Durante os 6,5 anos de seguimento do estudo, a taxa de filtração glomerular (TFG) declinou mais rapidamente em portadores do alelo Q (QQ/KQ) do que em não-portadores. As taxas médias de declínio foram 7,2 e 3,7 $ml.min^{-1}.ano^{-1}$, respectivamente. Com essa perda rápida da função renal, pacientes com DM portadores do alelo Q parecem progredir do estágio de proteinúria ao estágio de DRCT em menos anos do que os não-portadores. Além disso, o efeito desta variante foi mais evidente quando a DRCT se desenvolveu mais cedo no curso do DM1 (34).

Canani *et al*. (10), em um estudo de caso-controle e baseado em famílias, relatou que o alelo Q foi observado em 21,5% do grupo controle (sem DRD), 31,5% dos casos com proteinúria e 32,2% dos casos com DRCT (p = 0,012). Após estratificação por tempo de DM (duração de < 24 anos *vs*. ≥ 24 anos), o risco de desenvolver DRCT mais precocemente para os portadores do alelo Q foi 2,3x maior do que o risco para os não-portadores (IC 95% 1,2 - 4,6). Entretanto, alguns estudos posteriores não observaram associação do polimorfismo K121Q com DRD (**Tabela 1**), o que pode ser devido à falta de poder estatístico. Dessa forma, fazem-se necessários estudos adicionais que esclarecem a relevância do polimorfismo K121Q na suscetibilidade a essa complicação crônica do DM.

Tabela 1. Estudos de associação entre o polimorfismo K121Q no gene *E-NPP1* e doença renal do diabetes.

Autor, ano (ref.)	População de estudo	Nº de participantes	Resultados
Wu *et al*. 2009 (35)	População tailandesa com DM2	394	Associação entre o genótipo Q/Q e risco para DRD (RC = 1,85, IC 95% 1,17 – 2,92).
Keene *et al*. 2008 (36)	População afro-americana com DM2	577	Sem associação com DRD.
De Cosmo *et al*. 2000 (34)	Caucasianos com DM1 e proteinúria	77	Associação entre o alelo Q e diminuição da TFG (RC = 5,7, IC 95% 4,1 – 7,2).
Canani *et al*. 2002 (10)	Caucasianos com DM1	659	Associação entre o alelo Q e risco de DRCT (RC = 2,3, IC 95% 1,2 – 4,6).
De Cosmo *et al*. 2008 (37)	População americana e italiana com DM2	1.392	Associação entre o alelo Q e redução da TFG em pacientes de Gargano (RC = 1,69, IC 95% 1,1 – 2,6) e Boston (RC = 1,50, IC 95% 1,0 – 2,2).
Jacobsen *et al*. 2002 (38)	População dinamarquesa com DM1	295	Sem associação com DRD.
De Azevedo *et al*. 2002 (39)	População sul-americana com DM1	30	Sem associação com DRD.
Tarnow *et al*. 2001 (40)	Pacientes dinamarqueses com DM1	240	Sem associação com DRD.
Leitão *et al*. 2008 (41)	Pacientes com DM2 com descendência europeia e africana	1267	Sem associação com DRD.
Lin *et al*. 2011 (42)	Pacientes de Taiwan com e sem DRD	416	Associação entre o alelo Q e DRD (RC = 1,81, IC 95% 1,2 – 2,7).

DRD = doença renal do diabetes; TFG = taxa de filtração glomerular; DM1 = diabetes mellitus tipo 1; DM2 = diabetes mellitus tipo 2; IC = intervalo de confiança; RC = razão de chances.

A ASSOCIAÇÃO DAS ECTONUCLEOTIDASES COM REJEIÇÃO DO TRANSPLANTE RENAL

Mesmo que o tratamento da DRD envolva medidas para retardar a evolução da albuminúria e a perda de função renal, muitos pacientes ainda assim progridem até DRCT, necessitando de terapia de substituição renal (43, 44). Neste contexto, o transplante renal representa atualmente a melhor opção terapêutica para pacientes com DRCT, uma vez que está associado a uma melhor qualidade e expectativa de vida do que o tratamento dialítico (45, 46). Apesar da grande melhora que ocorreu nas últimas décadas nas taxas de sobrevida do enxerto renal, a sobrevida dos pacientes transplantados renais ainda é significativamente menor do que indivíduos da população em geral devido a fatores relacionados aos receptores e ao transplante [imunossupressão, ocorrência de infecções ou rejeição aguda (RA)] (47-49).

A RA é uma resposta imune destrutiva contra o enxerto que pode ocorrer a qualquer tempo durante a vida do órgão transplantado (50-54). O reconhecimento dos episódios de RA continua a ser um grande desafio a ser vencido para melhorar a sobrevida do enxerto, uma vez que a sua ocorrência está relacionada à progressão para disfunção crônica do enxerto, que é a causa mais frequente de falha do enxerto renal (55, 56). Segundo registros do *United Network for Organ Sharing* (UNOS), receptores de um primeiro transplante que não desenvolveram rejeição apresentaram uma taxa de sobrevida do enxerto, em um ano, de 86%, comparada com 67% naqueles com um ou mais episódios de rejeição (57-59).

Falhas no reconhecimento precoce de episódios de RA ou, da mesma forma, um diagnóstico presumido de RA onde existe a necessidade de tratamento imunossupressor agressivo, quando não confirmado, adicionam danos ao enxerto e ao paciente. Portanto,

torna-se indispensável à utilização de técnicas invasivas para o diagnóstico preciso da RA, como a biópsia renal, que é atualmente considerada o método de referência.

A identificação do grau de severidade das agressões ao enxerto é classificada segundo os critérios de Banff (60). A classificação de Banff se dá de acordo com critérios temporais (rejeição hiperaguda, aguda ou crônica), fisiopatológicos (celular-intersticial, vasculares, anticorpo-endotelial) ou de acordo com a gravidade (grau de inflamação histológica e lesão, como marcados e calibrados por meio do esquema de *Banff*), resposta ao tratamento (presença ou ausência de resistência ao tratamento com glucocorticóides), presença ou ausência de disfunção renal (indicando RA subclínica) ou mecanismos imunológicos envolvidos (resposta do sistema imune adaptativo ou inato) (61-64).

A RA é mediada por uma variedade de processos imunológicos e está associada a diversos fatores de risco, tais como a idade dos pacientes transplantados, compatibilidade do sistema HLA e ocorrência de função tardia do enxerto (*delayed graft function* – DGF) (50, 54). Um melhor entendimento desses mecanismos fisiopatológicos que levam à RA poderá aprimorar a sua detecção e monitorização, consequentemente, levando a melhora da sua prevenção e tratamento (57, 65, 66).

Considerando que a biópsia renal é um procedimento invasivo, com potencial de morbidade e sujeito a erro de amostragem, o desenvolvimento de métodos acurados e não invasivos para detecção precoce de RA tem sido objeto de estudos por vários grupos de pesquisadores (67, 68). Nesse cenário, diversos estudos têm usado técnicas de biologia molecular para identificar possíveis biomarcadores para o diagnóstico e/ou avaliação do prognóstico da RA (69-72). Entretanto, a maioria desses estudos que buscaram possíveis biomarcadores para RA foram realizados após o transplante.

Acreditamos, no entanto, que um teste preditivo pode ter um maior valor clínico se puder antever o risco de o paciente desenvolver RA antes mesmo da realização do transplante, o que poderia levar a escolha de estratégias imunossupressoras mais eficazes. Sendo assim, polimorfismos de DNA em genes candidatos à patogênese da RA constituem biomarcadores ideais, pois são de fácil coleta e análise, podendo ser genotipados em pacientes nas filas de espera para transplante.

Como algumas ectonucleotidases têm sido envolvidas em mecanismos de dano renal, processos inflamatórios e rejeição crônica (73-75), proteínas dessa família podem ser potenciais biomarcadores para RA. De fato, a NTPDase 1/CD39 já foi associada à modulação dos processos de injúria vascular de enxertos de tecido cardíaco em camundongos transplantados (76). A ecto-ATPase e a ecto-5'-nucleotidase foram envolvidas na falência do transplante renal em camundongos. A ecto-ATPase, bem como a ecto-AMPase, também foram relacionadas à rejeição crônica em pacientes transplantados renais (75). Até o momento, nenhum estudo avaliou se a E-NNP1 está envolvida na patogênese da RA ou se polimorfismos nessa proteína podem ser biomarcadores dessa complicação.

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3 OBJETIVOS

Avaliar a associação entre o polimorfismo K121Q no gene *E-NPP1* com DRD em pacientes diabéticos, bem como RA em pacientes transplantados renais do sul do Brasil.

OBJETIVOS ESPECÍFICOS

- Realizar revisão sistemática e metanálise de todos os estudos disponíveis na literatura para avaliar a associação entre o polimorfismo K121Q no gene *E-NPP1* e DRD em pacientes diabéticos;

- Avaliar a associação entre o polimorfismo K121Q no gene *E-NPP1* com RA em pacientes transplantados renais brancos.

4 CAPÍTULO I

Association between the *E-NPP1* K121Q polymorphism and risk of diabetic kidney disease: a systematic review and meta-analysis.

Artigo publicado na *PLOS One* 2015; volume 10 (3): e0118416

PLOS ONE

RESEARCH ARTICLE

Association between the *ENPP1* K121Q Polymorphism and Risk of Diabetic Kidney Disease: A Systematic Review and Meta-Analysis

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OPEN ACCESS

Citation: Sortica DA, Buffon MP, Souza BM, Nicoletto BB, Santer A, Assmann TS, et al. (2015) Association between the *ENPP1* K121Q Polymorphism and Risk of Diabetic Kidney Disease: A Systematic Review and Meta-Analysis. PLoS ONE 10(3): e0118416. doi:10.1371/journal.pone.0118416

Academic Editor: Alberico Catapano, University of Milan, ITALY

Received: October 10, 2014

Accepted: January 16, 2015

Published: March 20, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Fundo de Incentivo à Pesquisa e Eventos (FIPE) at the Hospital de Clínicas de Porto Alegre. D.C. and L.H.C. are recipients of scholarships from CNPq. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

The potential association between the K121Q (A/C, rs1044498) polymorphism in the ecto-nucleotide pyrophosphatase/phosphodiesterase (*ENPP1*) gene and risk of diabetic kidney disease (DKD) has been investigated. Nevertheless, the effect of this variant on DKD risk is still under debate, and conflicting results have been reported. To this date, no meta-analysis has evaluated the association of the K121Q polymorphism with DKD. This paper describes the first meta-analysis conducted to evaluate whether the *ENPP1*K121Q polymorphism is associated with DKD. A literature search was conducted to identify all case-control or cross-sectional studies that evaluated associations between the *ENPP1*K121Q polymorphism and DKD. Pooled odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for allele contrast, additive, dominant and recessive inheritance models. Seven studies were eligible for inclusion in the meta-analysis, providing data on 3571 type 1 or type 2 diabetic patients (1606 cases with DKD and 1965 diabetic controls without this complication). No significant heterogeneity was observed among the studies included in the meta-analysis when assuming different inheritance models ($I^2 < 50\%$ or $P > 0.10$ for the entire sample and after stratification by ethnicity). Meta-analysis results revealed significant associations between the K121Q polymorphism and risk of DKD in Asians and Europeans when assuming the different inheritance models analyzed. The most powerful association was observed for the additive model (OR = 1.74, 95% CI 1.27-2.38 for the total sample). In conclusion, the present meta-analysis detected a significant association between the *ENPP1*K121Q polymorphism and increased susceptibility of DKD in European and Asian populations.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

The number of people living with diabetes mellitus (DM) worldwide is expected to double between 2000 and 2030 [1,2]. This global increase in the prevalence of DM will lead to a higher incidence of the chronic complications of diabetes [3]. Diabetic kidney disease (DKD) is a common diabetic chronic complication [4]. DKD is characterized by albuminuria and reduced glomerular filtration rate (GFR) [4]. Approximately 40% of patients with diabetes, whether type 1 or type 2, develop some degree of renal disease after many years of DM [5]. DKD is thus the most common cause of end-stage renal disease in several countries, and it is associated with high morbidity and mortality rates among diabetic patients [6,7].

The mechanisms involved in the pathogenesis of DKD are multiple and complex. Both epidemiological and familial studies have shown major agreement for clustering of DKD in some families, strengthening the hypothesis that there are important genetic factors involved in its pathogenesis [7]. Environmental factors such as hyperglycemia, arterial hypertension and/or dyslipidemia are known to play a key role in the development of DKD in genetically susceptible subjects [5,6,8,9]. Therefore, extensive efforts have been made to identify which genes are related to DKD; however, results are still inconclusive due to several genes being associated with small effects in different populations [10,11,12,13]. The identification of such genes will help detect individuals at high risk of developing DKD, and might provide a better understanding of its pathophysiology [14].

Candidate genes for insulin resistance (IR) can also be considered as DKD candidate genes in patients with type 1 or type 2 DM [15]. In this context, the gene that encodes the ectonucleotide pyrophosphatase/phosphodiesterase (ENPP1) is a good candidate gene for DKD [12,16]. The *ENPP1* gene is expressed in many tissues, including the kidneys, and it is known that increased *ENPP1* gene expression blockades the tyrosine kinase activity of the insulin receptor in several cells, causing IR [7]. More than 15 years ago, a polymorphism was reported in exon 4 of this gene leading to a lysine (K) to glutamine (Q) substitution in codon 121 (K121Q; rs1044498) [7,12]. This change is located in one of the ENPP1 somatomedin-B-like domain, and might influence protein-protein interactions [7,17]. Furthermore, the Q allele of the K121Q variant has been shown to influence ENPP1 protein function by inhibiting insulin receptor function and insulin signaling more effectively than the K allele [12].

Since then, several studies have investigated the association between the K121Q polymorphism and IR, type 2 DM (T2DM) and/or its chronic complications, such as DKD (for a review, see [7]). However, the impact of this polymorphism on DKD susceptibility is still under debate, with contradictory findings: while some studies reported an association of the Q allele with DKD [18,19,20,21], other studies were not able to replicate this association [22,23].

Thus, to further investigate the potential association of the *ENPP1* K121Q polymorphisms with DKD, we conducted a systematic review and meta-analysis of the literature on the subject.

Materials and Methods

Search strategy and eligibility criteria

This study was designed and reported in agreement with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), Meta-analysis of Observational Studies in Epidemiology (MOOSE) statements, and following the Meta-analysis on Genetic Association Studies Checklist from Plos One (**S1 Table**) [24,25].

PubMed and Embase databases were searched systematically to identify all genetic studies that investigated associations between DKD and the K121Q polymorphism. The K121Q polymorphism was selected for the present meta-analysis because it has been the most frequently

studied polymorphism in *ENPP1* gene. There are not enough data concerning the other *ENPP1* polymorphisms and diabetic kidney disease to perform a meta-analysis. The following medical subject headings (MeSH) were used: ("Phosphodiesterase I"[7] OR "ectonucleotide pyrophosphatase phosphodiesterase 1"[Supplementary Concept]) AND ("Polymorphism, Genetic"[Mesh] OR "Polymorphism, Single Nucleotide"[Mesh] OR "Polymorphism, Restriction Fragment Length"[Mesh] OR "Amplified Fragment Length Polymorphism Analysis"[Mesh] OR "Polymorphism, Single-Stranded Conformational"[Mesh] OR "DNA Copy Number Variations"[Mesh] OR "Mutation"[Mesh] OR "Mutation, Missense"[Mesh] OR "INDEL Mutation"[Mesh] OR "Point Mutation"[Mesh] OR "Frameshift Mutation"[Mesh] OR "Codon, Nonsense"[Mesh]) AND ("Diabetes Mellitus"[Mesh] OR "Diabetes Complications"[Mesh] OR "Diabetes Mellitus, Type 2"[Mesh] OR "Diabetes Mellitus, Type 1"[Mesh]). The search was restricted to papers reporting on human subjects and published in English or Spanish, and was completed on July 25th, 2014. It is worth noting that although we aimed to analyze only studies published in English or Spanish, we did not identify any study in another language which analyzed the K121Q polymorphism and diabetic kidney disease. All of the papers found were also searched manually to identify other relevant citations. Moreover, unpublished results were searched in the abstract books of the Endocrine Society, American Diabetes Association, and European Association for the Study of Diabetes (EASD) Meetings.

Eligibility was evaluated through a review of titles and abstracts; when abstracts did not provide sufficient information, the full text of the paper was retrieved for analysis, as in previous reviews by our group [26,27,28]. Briefly, this was done independently in a standardized manner by two investigators (D.A.S and M.P.B.). Disagreements were resolved by discussion between them and, if required, a third reviewer (D.C.) was consulted. We included observational studies that evaluated the frequencies of the K121Q polymorphism in patients with DKD (cases) and diabetic patients without any degree of DKD (controls). Both type 1 and type 2 diabetic patients older than 18 years were included. Studies would be excluded from analysis if the genotype distributions in the control group deviated from those predicted by Hardy-Weinberg equilibrium (HWE) or if they did not provide sufficient data to estimate an odds ratio (OR) with 95% CI. However, no study was excluded due to these criteria. If results were duplicated and had been published more than once, the most complete article was included in the study.

Data extraction

Necessary information from each study was extracted by two investigators working independently (D.A.S. and M.P.B.), using a standardized extraction form, and consensus was sought for all extracted items. When consensus could not be achieved, differences in data extraction were decided by reading the original publication [26,27,28]. The data extracted from each study were as follows: (1) characteristics of the study (including name of the first author, year of publication, number of subjects included in the case and control groups) and sample characteristics, such as age, gender, ethnicity, type of DM, DM duration, HbA1c, body mass index (BMI), systolic and diastolic blood pressure, percentage of hypertension, lipid profile, DKD classification and information regarding kidney function; (2) case and control definitions; (3) polymorphism frequencies (including genotype and allele distributions in case and control groups and ORs with 95% CIs). When data were not available, the authors were contacted by email.

Quality control assessment

Two investigators (D.A.S. and D.C.) independently evaluated the quality of each eligible article using the Newcastle-Ottawa Scale (NOS) for observational studies [29]. The NOS contains

nine items subdivided into three dimensions, including selection, comparability, and exposure. For each item, a sequence of answer alternatives is provided. A star scoring system is used for semi-quantitative evaluation of article quality, such that the highest-quality studies are assigned a maximum of one star for each item, with the exception of the comparability item, which can be assigned two stars. Therefore, the total NOS score ranges from zero to nine stars.

Statistical analysis

Control subjects' genotype frequencies were tested for compliance with HWE using a goodness-of-fit chi-square (χ^2) test. Gene-disease associations were measured using OR (95% CI) estimations based on the following genetic inheritance models: 1) allele contrast; 2) additive model; 3) recessive model; 4) and dominant model [30]. Taking into account that the frequency of the *ENPP1* K121Q polymorphism varies across different populations, gene-disease associations for the different inheritance models were also analyzed according to ethnicity.

Heterogeneity was evaluated using a χ^2-based Cochran's Q statistic and inconsistency was tested by the I^2 metric. Heterogeneity would be considered statistically significant at $P < 0.10$ for the Q statistic or $I^2 > 50\%$ for the I^2 metric statistic (29, 30). However, since no significant heterogeneity was detected, the fixed effect model (FEM) was used to calculate OR (95% CI) for each individual study and for the pooled effect [31]. Due to the lack of inter-study heterogeneity, we did not perform meta-regression analyses (adjusting for covariables such as age, sex, body mass index or environmental factors) or sensitivity analysis. All statistical analyses were performed using Stata 11.0 software (StataCorp, College Station, TX, USA).

Results

Our search strategy yielded 115 possibly relevant papers (**Fig. 1**), 103 of which were excluded following the review of titles and abstracts. Twelve articles appeared eligible after this phase and were selected for full-text evaluation. Nevertheless, after cautious reading of the full texts, further studies were excluded owing to missing information, ineligible study design or because the *ENPP1* polymorphism reported was not the one of interest for this meta-analysis. Therefore, seven articles fulfilled the eligibility criteria and were included in the meta-analysis, providing data on 3571 subjects (1606 cases with DKD and 1965 diabetic controls without this complication). The study reported by Leitão *et al.* [10] was subdivided into two studies because it analyzed the *ENPP1* polymorphism in two different populations.

S2 Table depicts the main characteristics of the studies included in our meta-analysis. Regarding the DKD classification, Canani *et al.* [18] subdivided cases into two groups, proteinuric and end-stage renal disease (ESRD), while Leitão *et al.* [10] and De Cosmo *et al.* [20] defined DKD patients as microalbuminuric or macroalbuminuric. Wu *et al.* [19], Lin *et al.* [21] and Tarnow *et al.* [32] classified all DKD cases as macroalbuminuric. **S3 Table** lists genotype and allele distributions and ORs (95% CI) for the *ENPP1* K121Q polymorphism in case and control groups from the five articles reviewed.

A quality evaluation of each individual study included in the meta-analysis is shown in **Table 1**. The highest-quality papers were awarded nine stars. Overall, most studies were classified as having at least moderate quality in terms of selection, comparability and exposure criteria. Wu *et al.* [19] was awarded eight stars; Lin *et al.* [21], Canani *et al.* [18] and De Cosmo *et al.* [20] were awarded seven stars; Tarnow *et al.* [32], six stars; and Leitão *et al.* [10], five stars.

Table 2 summarizes the results of the pooled meta-analyses for associations between the *ENPP1* K121Q polymorphism and risk of DKD. In general, our results revealed significant associations between the K121Q polymorphism and risk of DKD when assuming allele contrast, additive, recessive and dominant inheritance models. Notably, the most powerful association

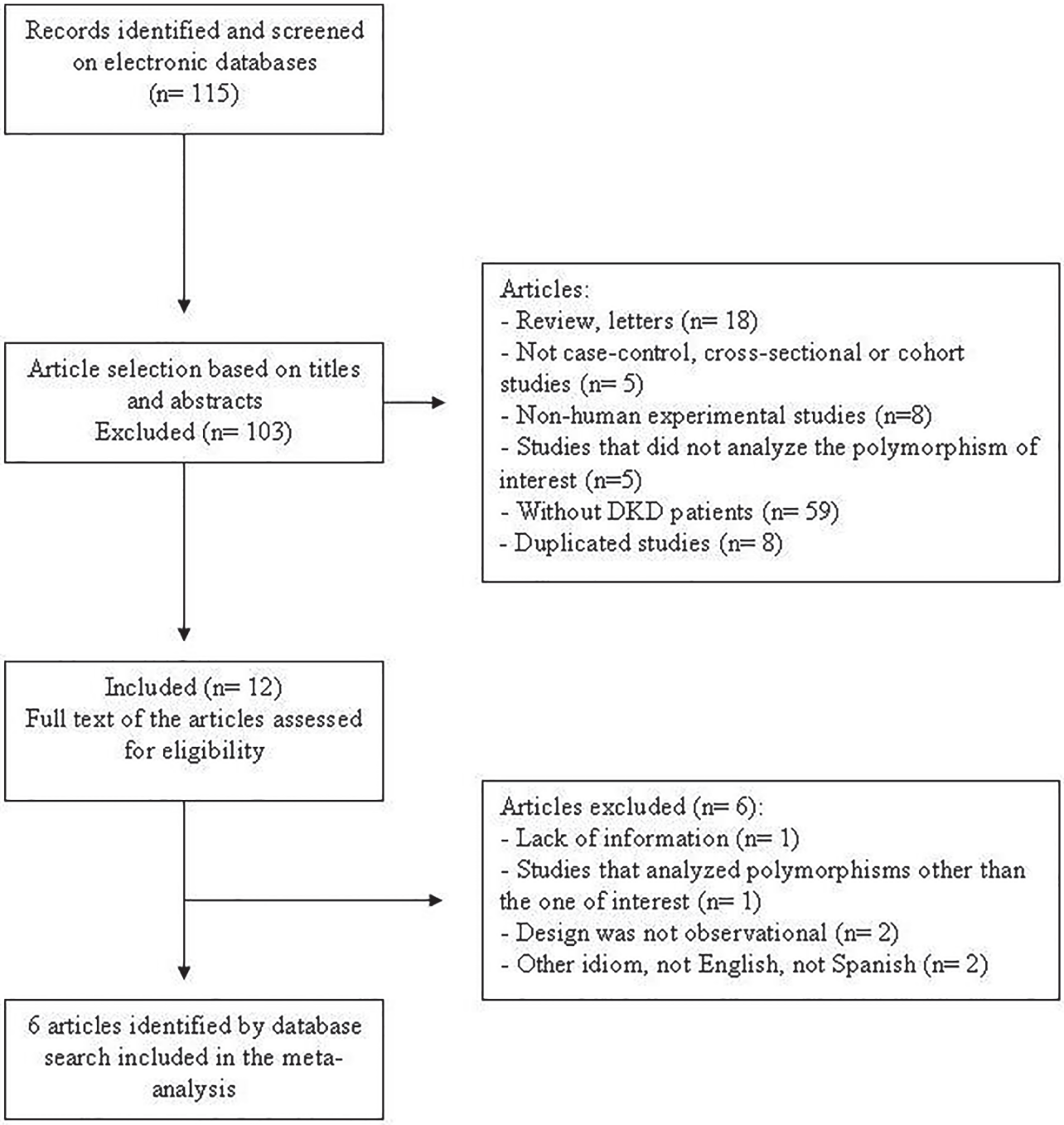

Fig 1. Flowchart illustrating the search strategy used to identify studies of association between the *ENPP1* K121Q polymorphism and diabetic kidney disease for inclusion in the meta-analysis.

doi:10.1371/journal.pone.0118416.g001

was observed for the additive model (OR = 1.74, 95% CI 1.27–2.38). Moreover, after stratification by ethnicity, the associations between the K121Q polymorphism and risk of DKD remained in Europeans and Asians (**Table 2 and Fig. 2**). There was only one study performed in Africans [10], which showed that the K121Q polymorphism was not associated with DKD in this population.

Table 1. Newcastle-Ottawa quality assessment scores for the studies included in the meta-analysis.

Author	Year	Selection	Comparability	Exposure
Tarnow et al. [32]	2001	**	**	**
Canani et al. [18]	2002	***	*	***
Leitão et al. [10]	2008	**		***
De Cosmo et al. [20]	2009	***	**	**
Wu et al. [19]	2009	***	**	***
Lin et al. [21]	2011	**	**	***

doi:10.1371/journal.pone.0118416.t001

No significant heterogeneity was observed among the analyzed studies investigating the *ENPP1* K121Q polymorphism when assuming different inheritance models ($I^2 < 50\%$ or $P > 0.10$ for the entire sample and also after stratification by ethnicity; **Table 2**).

Discussion

The effect of the *ENPP1* K121Q polymorphism on susceptibility to DKD is still controversial: while some studies reported an association of the Q allele with DKD risk [18,19,20,21,33], other studies were not able to replicate this association [22,23], possibly due to small sample sizes and differences in K121Q frequencies among ethnicities. Therefore, to further evaluate whether this polymorphism is associated with DKD risk, we conducted a meta-analysis of five

Table 2. Pooled measures for association between the *ENPP1* K121Q polymorphism and susceptibility to diabetic kidney disease.

Inheritance model	No. of studies	No. of cases	I^2 (%)	Pooled OR (95% CI)
Allele contrast				
Overall*	7	1,606	41.0	1.36 (1.20–1.53)
European	4	1,102	17.9	1.28 (1.11–1.49)
Asian	2	431	0.0	1.72 (1.35–2.18)
Additive				
Overall*	7	1,606	10.5	1.74 (1.27–2.38)
European	4	1,102	30.4	1.79 (1.16–2.76)
Asian	2	431	0.0	2.15 (1.23–3.75)
Recessive				
Overall*	7	1,606	5.5	1.54 (1.15–2.08)
European	4	1,102	31.8	1.71 (1.11–2.62)
Asian	2	431	0.0	1.75 (1.01–3.02)
Dominant				
Overall*	7	1,606	43.7	1.40 (1.21–1.62)
European	4	1,102	27.2	1.28 (1.08–1.52)
Asian	2	431	0.0	1.91 (1.43–2.55)

No significant heterogeneity was observed among the analyzed studies investigating the *ENPP1* K121Q polymorphism when assuming different inheritance models. Thus, fixed effect models (FEM) were used for the calculation of OR (95% CI) for each individual study and for the pooled effect.
* Studies included: 4 with patients of European descent; 2 with patients of Asian descent; 1 with patients of African descent.

doi:10.1371/journal.pone.0118416.t002

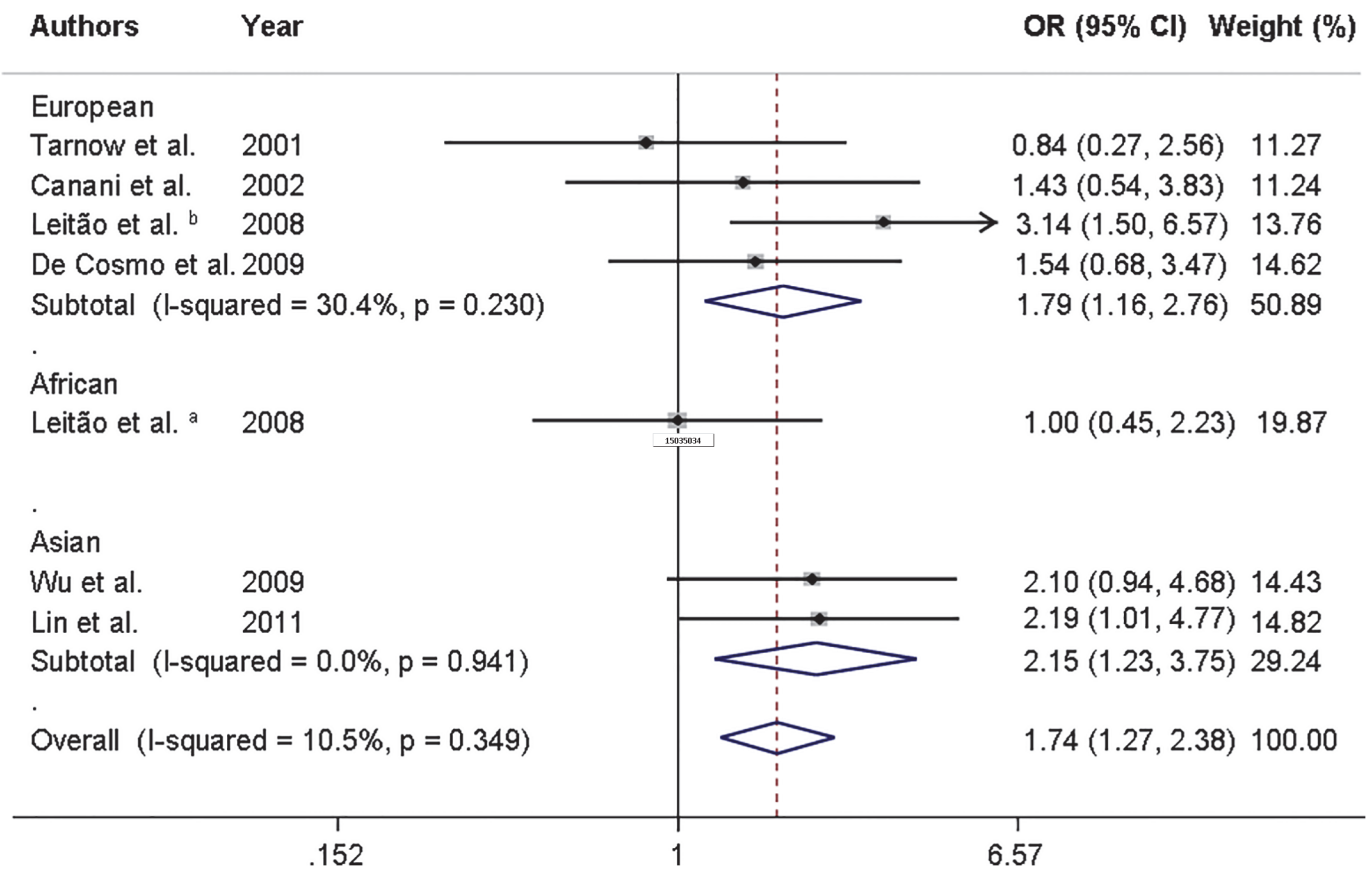

Fig 2. Forest plot showing individual and pooled ORs (95%CI) for the association between the *ENPP1* K121Q polymorphism and diabetic kidney disease after stratification by ethnicity, under an additive inheritance model. The area of the squares reflects the study-specific weight. The diamond illustrates the fixed-effect model summary OR (95% CI).

doi:10.1371/journal.pone.0118416.g002

studies carried out in different populations. Our results revealed significant associations between the K121Q polymorphism and risk of DKD in all genetic inheritance models analyzed.

As already mentioned, increased ENPP1 levels blockade the tyrosine kinase activity of the insulin receptor α-subunit in many cells, causing IR [7,34,35]. Consequently, ENPP1 has being associated with impaired glucose metabolism. Subjects with IR have high concentrations of ENPP1 protein [7,36,37,38,39]. Accordingly, transgenic mice overexpressing *ENPP1* in skeletal muscles and liver showed elevated glucose and insulin concentrations as well as decreased glucose uptake in muscle [40]. In contrast, the total knockdown of *ENPP1* gene in the mice liver was able to decrease both postprandial and fasting plasma glucose levels [7,41].

Thus, taking into account the role of ENPP1 in IR, several studies have investigated the association between the *ENPP1* K121Q polymorphism and IR or T2DM (reviewed in [7]). To this date, eight meta-analyses had reported that *ENPP1* 121Q allele carriers in different populations and ethnicities are at increased risk of developing T2DM [11,42,43,44,45,46,47,48]. In contrast, meta-analyses performed by Zhao *et al.* [49] and Weedon *et al.* [50] did not find such an association with T2DM in Europeans.

Regarding the association between the K121Q polymorphism and DKD or related features, De Cosmo *et al.* [51] showed that this variant influences GFR decline in proteinuric type 1 diabetic patients: GFR decreased earlier in subjects with QQ/KQ genotypes compared to subjects with KK genotype. We previously demonstrated that the Q variant was associated with ESRD

in type 1 diabetic patients and short DM duration [18]. Q allele carriers (KQ/QQ) had a two fold increased risk of developing ESRD when compared to patients with KK genotype (OR 2.3; 95% CI 1.2–4.6) [18]. Moreover, De Cosmo *et al.* [20] reported that Italian T2DM patients with the Q allele had an increased risk of having a decreased GFR as well as more severe DKD than non-carriers. This is in accordance with data reported by Lin *et al.* [21] and Wu *et al.* [19], who also described an association between the Q allele and increased risk of DKD in Asian patients with T2DM. Nevertheless, other studies did not observe any association between the K121Q polymorphism and DKD or related features [7,10,23,32]. This might be due to an effect of ethnicity on this association. The present meta-analysis suggests that the Q allele is significantly associated with DKD in both Europeans and Asians, possibly under an additive inheritance model. There was only one [10] study that included African descent subjects. Therefore, the role of this polymorphism in these subjects remains to be determined. Taking into account the small number of studies carried out in each ethnicity, additional studies with larger sample sizes are still needed to confirm the association between the K121Q variant and DKD in different ethnicities.

It is worth noting that several genome wide association studies have searched for chromosomal regions linked or associated with renal function phenotypes in T2DM patients, such as DKD, eGFR or creatinine/albumin ration [52,53,54,55,56,57,58,59,60,61]. The *ENPP1* gene is located in the 6q22.q23 region (http://www.ncbi.nlm.nih.gov/gene). Therefore, although some studies reported associations between polymorphic markers on chromosome 6q with DKD phenotypes [53,56,59], the reported closest region to 6q22-q23 was an association of a marker on 6q22.31 with survival on dialysis rates in African-Americans T2DM patients [61]. Moreover, Mooyaart *et al.* [62] performed a meta-analysis to assess the pooled effects of several genetic variants that have reproducibly been associated with DKD in previous studies. Their search identified 34 replicated genetic variants and, of these, 21 remained associated with DKD in the meta-analysis. Importantly, the K121Q polymorphism was not included among the 34 identified genetic variants since the results regarding this polymorphism were not constantly replicated in different populations.

The specific mechanisms that explain the association between the Q allele and risk for DKD are not known [7]. However, it is biological plausible that ENPP1 has a role in kidney tissue injury since it is acknowledged that *ENPP1* gene is expressed in both kidney mesangial and endothelial cells [63], and these cells show progressive pathological changes during the progression from normoalbuminury to overt DKD [7,18]. The Q allele interacts more strongly with the insulin receptor than the K allele, decreasing the autophosphorylation of this receptor [12]. The Q allele carriers seem to exhibit worse IR and hyperinsulinemia than subjects with the KK genotype [64]. Hyperinsulinemia might increase sodium resorption in the kidneys, causing augmented sympathetic-adrenergic activity, volume expansion, and increased expression of the angiotensin type II receptor, impairing peripheral vasodilatation [65]. Reduced vasodilatation as well as increase volume might predispose to arterial hypertension, a well known risk factor for DKD [7,65,66].

Meta-analysis has been regarded as a powerful tool for pooling data from several studies, which could overcome the problem of small sample numbers as well as insufficient statistical power of genetic association studies of complex diseases [26]. Of note, the present meta-analysis had an 80% power ($\alpha = 0.05$) to detect an OR $\geq$1.35. Nevertheless, the results of the present meta-analysis should be interpreted within the context of a few limitations. Meta-analyses can be prone to publication bias, and although we made every effort to find unpublished results, we cannot be sure if small negative studies were overlooked. One of the studies identified [23] was not included in our meta-analysis because its control group was constituted of healthy subjects without DKD, and our inclusion criteria were restricted to studies that comprised control

subjects with DM but without any degree of DKD. Keene *et al.* [23] did not observe any association between the K121Q polymorphism and ESRD in T2DM patients from an African-American population. In addition, heterogeneity could be a significant problem when interpreting the findings of any meta-analysis. In short, within these limitations, our data seem to be robust, since we did not detect any significant inter-study heterogeneity in any of the genetic inheritance models analyzed.

In conclusion, our results indicate that the *ENPP1* K121Q polymorphism is associated with risk of DKD in European and Asian populations. Since only small sample sizes could be obtained for analyses stratified by ethnicity, further studies with larger sample sizes are needed to confirm the effect possibly played by ENPP1 in the pathogenesis of DKD and related features.

Supporting Information

S1 PRISMA Checklist.
(DOC)

S1 Table. Meta-analysis on genetic association studies checklist.
(DOCX)

S2 Table. Characteristics of the studies included in the meta-analysis. Legend: NA: not evaluated. DKD: Diabetic Kidney Disease. 1: Urinary albumin excretion (UAE) (mg/24h): 796(16-14545). 2: Albumin Creatinine Ratio (ACR): 250 mg/g (men) or 355 mg/g (women). 3: Microalbuminuria: UAE 20–199 μg/min or Proteinuria: UAE ≥200 μg/min. 4: Microalbuminuria: UAE 20–199 μg/min or Proteinuria: UAE ≥200 μg/min. 5: Positive dipstick test for protein; Macroalbuminuria: two tests of spot urinary albumin >300 mg/mg of creatinine. 6: ACR >300 μg/mg; Blood Urea Nitrogen >20 mg/dl or Creatinine >1.7 mg/dl.
(DOC)

S3 Table. Genotype and allele distributions of the *ENPP1* K121Q polymorphism in patients with diabetic kidney disease and control subjects. Legend: [a] African descendants; [b] European descendants.
(DOC)

Author Contributions

Conceived and designed the experiments: DAS BMS TSA DC. Performed the experiments: DAS MPB BMS BBN AS TSA. Analyzed the data: DAS BMS AS TSA DC. Contributed reagents/materials/analysis tools: LHC. Wrote the paper: DAS BBN DC LHC.

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5 CAPÍTULO II

***Ectonucleotide Pyrophosphatase/Phosphodiesterase 1* K121Q polymorphism is associated with acute kidney rejection.**

***Ectonucleotide Pyrophosphatase/Phosphodiesterase 1* K121Q polymorphism is associated with acute kidney rejection**

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Running Title: *E-NPP1* K121Q polymorphism in kidney graft rejection

Abstract

The identification of risk factors for acute rejection may lead to strategies to improve success of kidney transplantation. Some proteins of the Ectonucleotidase family have been linked to transplant rejection processes. However, the association of Ectonucleotide pyrophosphatase/phosphodiesterase 1 (E-NPP1) with acute rejection has not yet been evaluated. Therefore, the aim of this study was to evaluate the association between the K121Q (rs104498) polymorphism in the *E-NPP1* gene and acute rejection in kidney transplant patients. The study comprised 454 subjects from a retrospective cohort of 613 kidney transplant patients in South of Brazil. Demographic and clinical data were collected, and the K121Q polymorphism was genotyped by real-time PCR using TaqMan MGB probes. Cox regression analysis was used to evaluate freedom of AR episodes in kidney transplant patients according to the different K121Q genotypes. The Q allele frequency was 17.0% in non-acute rejection patients and 24.5% in acute rejection patients (P = 0.024). Genotype frequencies of the K121Q polymorphism were in Hardy-Weinberg Equilibrium in controls (P = 0.810). The Q/Q genotype was associated with acute rejection [HR = 2.76, 95% CI 1.44 – 5.30, P = 0.002], adjusting for number of pregnancies and transfusions, delayed graft function, donor and recipient age and HLA-DR mismatches. Our findings suggest, for the first time, an association between the *E-NPP1* 121Q/Q genotype and acute rejection in kidney transplant patients.

Introduction

Kidney transplantation has become the treatment of choice for the majority of the patients with end-stage renal disease since it has been shown to provide better quality of life and life expectancy than dialysis treatment (1, 2). However, the survival rate in kidney transplant recipients is significantly lower than age-matched controls in the general population due to factors related to the recipient (previous illness, dialysis treatment) and to the transplantation process [immunosuppression, delayed graft function (DGF), infections and acute rejection (AR)] (3-5). AR episodes are related to progression to chronic graft dysfunction, which is the most prevalent cause of renal transplant failure (6).

Acute rejection is an allograft destructive immune response that may occur at any time during the life-span of a transplanted organ. A number of factors are known to be associated with increased risk of AR, including recipient's age, HLA sensitization and occurrence of DGF. However, these factors alone do not fully explain all cases of rejection (7, 8). The gold standard for AR diagnosis is the allograft biopsy, which presents some risks related to the procedure and is only useful once rejection has already occurred, being unable to predict rejection (9). Thus, the development of noninvasive tools able to predict risk for AR might be of great interest towards the improvement of allograft survival (10-12). In this scenario, some DNA polymorphisms might be predictors of AR and deserve to be further investigated (13).

Ectonucleotidases are ectoenzymes that hydrolyze extracellular nucleotides into nucleosides. They consist of four families, namely, ectonucleoside triphosphate diphosphohydrolases (NTPDases), ectonucleotide pyrophosphatase/phosphodiesterases (NPPs), ecto-5'-nucleotidases and alkaline phosphatases, and they are involved in the

modulation of purinergic signaling (14). Different ectonucleotidases have been linked to transplant chronic rejection processes, including NTPDase1 (15) and ecto-5'-nucleotidase (16).

Ectonucleotide pyrophosphatase/phosphodiesterase 1 (E-NPP1) belongs to the NPP family. This enzyme was first reported as a surface marker of B lymphocytes, being previously called plasma-cell differentiation antigen-1 (PC-1). E-NPP1 is a cell membrane protein with an extracellular active site catalyzing the release of nucleoside 5-monophosphatase from nucleotides and their derivatives (17). This enzyme is expressed in various tissues, including kidney, heart, brain, pancreatic islets, placenta, lung, salivary gland, epididymis, chondrocytes and lymphocytes (17).

E-NPP1 seems to be involved in immune system modulation (18, 19), possibly via degradation of extracellular adenosine triphosphate (ATP) and adenosine generation (20). It has been shown that the K121Q polymorphism in the *E-NPP1* gene is a risk factor for diabetes mellitus (DM) (17, 21) and diabetes kidney disease (22, 23), but its association with immune system or transplant outcomes has not yet been studied.

Taking into account the involvement of different ectonucleotidases in transplant rejection, and the association of E-NPP1 with immune modulation, we hypothesized that polymorphisms in the *E-NPP1* gene might be involved in AR process and, if so, could be used as predictor markers for AR. Thus, the aim of this study was to evaluate the association between the *E-NPP1* K121Q polymorphism and AR in kidney transplant recipients.

Methods

Design and patients

This nested case-control study was undertaken within a cohort of kidney transplant recipients from Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil), and was designed in accordance with STROBE and STREGA guidelines for reporting of genetic association studies (24, 25). Six hundred and thirteen kidney transplant patients were initially recruited from 2002 to 2016. All recipients were followed-up for at least one year post-transplantation. Among them, 501 (81.7%) patients were self-defined as white. Considering that the frequency of the *E-NPP1* K121Q polymorphism differs between ethnic groups (26, 27), we excluded non-white subjects from the study. We also excluded patients younger than 18 years old, without genotype data and those who received previous transplantation, corresponding to 9.2% of the total group. Hence, the analyzed sample comprised a total of 454 subjects. Among them, 96 patients had, at least, one episode of AR (cases) and 358 patients did not have AR diagnosed during follow-up (control group) (**Supplementary Figure 1**).

Patients were classified as having AR according to Banff classification (28) by an experienced pathologist. We investigated all the rejection episodes that occurred in this population during the first year of transplantation. Pathology data were collected retrospectively from kidney transplant electronic records. The following demographic and clinical data were also collected: age at transplantation, donor age, gender, primary kidney disease, family history of DM, blood pressure (BP), smoking, donor specific antibodies (DSA), number of blood transfusions and pregnancies, dialysis modality and duration, retransplant number, HLA mismatches, panel reactive antibody (PRA), donor type, cytomegalovirus or hepatitis C virus (HCV), DGF (defined by the requirement for

hemodialysis in the first post-transplantation week), type of donor (living or deceased), immunosuppressive regime, time after transplantation at the moment of evaluation, and time of AR diagnosis. Peripheral blood samples were collected from all patients for DNA extraction and genotyping of the *E-NPP1* K121Q polymorphism.

The study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre, and all subjects received adequate information about this study and gave their written informed consent.

HLA typing

Until 2006, HLA phenotyping was done using the PCR-SSP (polymerase chain reaction - sequence specific primers) technique (29, 30). Briefly, PCR-amplified DNA fragments were separated by electrophoresis in 1.5% agarose gels and visualized by staining with ethidium bromide and exposure to UV light. Interpretation of PCR-SSP results was based on the presence or absence of a specific amplified DNA fragment. After 2006, HLA phenotyping was performed using PCR-SSO (PCR - sequence specific oligonucleotide) (31). In this method, the target DNA was amplified by PCR using a specific primer, and then, the PCR product was denatured and allowed to hybridize to complementary DNA probes coupled with fluorescence-encoded beads. A flow analyzer identifies the fluorescence intensity in each microsphere, and the assignment of HLA typing was based on the reaction pattern compared with published sequences of HLA genes (32).

Genotyping of the E-NPP1 K121Q polymorphism

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. Genotyping of the K121Q (A/C) polymorphism (rs1044498) in

exon 4 of the *E-NPP1* gene was performed using primers and probes contained in the Human Custom TaqMan Genotyping Assay 20x (Thermo Fisher Scientific Inc., Waltham, MA, USA). Primer and probe sequences used for genotyping were: 5-AGCCTCTGTGCCTGTTCAG-3' (forward primer), 5'-ACACACAGAACTGTAGTTGATGCA-3' (reverse primer), 5'-AGTCGCCCTTGTCCTT-3' (VIC probe), and 5'-TCGCCCTGGTCCTT-3' (FAM probe). All reactions were conducted in 96-well plates, in a total of 5 μl volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Thermo Fisher Scientific Inc.) and Custom TaqMan Genotyping Assay 1x, and ran on the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.).

Statistical analyses

Allelic frequencies were determined by gene counting, and departures from the Hardy–Weinberg equilibrium (HWE) were verified using χ^2 test. Allele and genotype frequencies were compared between groups of patients using χ^2 tests. Clinical and laboratory characteristics were compared between groups by using unpaired Student's t-test or χ^2, as appropriate. Variables are presented as mean ± SD or number (%). The magnitude of association was estimated using odds ratios (ORs) with a 95% confidence interval (95% CI). Hazard ratio (HR) and 95% CI obtained from a Cox regression model was used to evaluate freedom of AR episodes in patients according to the presence of the 121Q/Q genotype. Results for which P values were less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 18.0 (SPSS, Chicago, IL).

Results

Sample description

Three hundred and fifty eight patients showed no episodes of AR (non-AR group), whereas 91 patients presented histologically proven acute cellular rejection and 5 patients showed acute humoral rejection, resulting in a total of 96 (21%) acute rejection episodes (AR group) among the 454 kidney transplant recipients included in this study.

Patient's demographics and clinical characteristics are shown in **Table 1**. In general, there were no significant differences between AR and non-AR groups regarding gender, donor age, number of blood transfusions, number of pregnancies, renal replacement therapy modality, donor type, cold ischemia time (CIT), hypertension, and PRA Class I and II. However, as expected, the mean of total HLA-mismatches was higher in the AR group than in the non-AR group (P = 0.008). In the same way, the frequency of patients with ≥2 HLA-DR mismatches was higher in the AR group than in the non-AR group (P = 0.009). Moreover, patients with AR were younger than the non-AR group (P = 0.038), and DGF occurred more frequently in the AR group compared to non-AR patients (P = 0.049). It is noteworthy that when stratifying patients according to the two techniques used for HLA immunophenotyping (as described in the Methods Section), this did not change the results (data not shown).

Molecular analyses

Genotype frequencies were in HWE in controls (P= 0.810). The presence of Q/Q genotype, assuming either additive (K/K *vs*. Q/Q; OR = 4.12; 95% CI 1.70 – 9.95; P = 0.002) or recessive (K/K+K/Q *vs*. Q/Q; OR = 4.08; 95% CI 1.71 – 9.73; P = 0.002)

inheritance models was associated with AR (**Table 2**). Accordingly, the Q allele frequency was increased in AR patients compared to non-AR patients (24.5% *vs*. 17.0%, respectively; P = 0.024).

Figure 1 illustrates that the Q/Q genotype is a risk factor for AR compared to K/K and K/Q genotypes (HR = 2.78, 95% CI 1.43 – 5.43, P= 0.003), adjusted for number of pregnancies and transfusions, DGF, donor and recipient age and HLA-DR mismatches.

Discussion

We have demonstrated, for the first time that the *E-NPP1* 121Q/Q genotype is independently associated with risk of developing AR in white kidney transplant patients. The precise mechanisms behind this finding are uncertain and need to be further elucidated.

Other ectonucleotidases have been shown to modulate local immune responses by lymphocytes, acting to reduce inflammation, possibly via degradation of the extracellular ATP and generation of adenosine (14, 20, 33, 34). Members of the NTPDase (CD39) family are cell membrane enzymes that hydrolyze ATP into adenosine diphosphate (ADP) as well as ADP into adenosine monophosphate (AMP) in three different steps, releasing inorganic phosphate (Pi). Then, ecto-5'-nucleotidases (CD73) dephosphorylate AMP into adenosine (35, 36). In contrast, E-NPP1 is able to degrade ATP and ADP into AMP in a single step, releasing AMP along with pyrophosphate (PPi) (14). In the final hydrolyzation step, the extracellular AMP can be hydrolyzed to adenosine and Pi by the effect of either ecto-5′-nucleotidase (CD73) or one of the four alkaline phosphatase isoforms (33, 35) (**Figure 2-A**).

NTPDase1 (CD39) and ecto-5'-nucleotidases (CD73), as major nucleotide metabolizing enzymes, are known to regulate immunity and inflammation, and possibly, to protect against hypoxic and ischemic tissue injuries (37). Accordingly, the combination of CD39 and CD73 can be viewed as "immunological switches" that shift ATP-driven pro-inflammatory immune cell activity toward an anti-inflammatory state mediated by adenosine (37). Poelstra *et al*. (38), studying a murine glomerulonephritis model, indicated that the ecto-5'-nucleotidase have anti-inflammatory activity in glomerular cells. Thus, considering the above-mentioned evidence, it is reasonable to suppose that E-NPP1, as part of the ectonucleotidase family, might play a role in the immune mechanisms that lead to AR of kidney grafts. In this context, the K121Q polymorphism might be decreasing E-NPP1 activity. Further experimental studies are needed in order to clarify the role of E-NPP1 in AR.

In order to speculate mechanistically our findings regarding the association of the *E-NPP1* K121Q polymorphism and AR, it is important to mention that adenosine, released by ectonucleotidase activities (including E-NPP1), is known to be an inhibitory mediator of T effector lymphocytes in various immune diseases (39-42). CD39 and C73 are expressed on the surface of T regulatory (Treg) cells, converting ATP into adenosine, which acts as a substrate for Treg immunosuppressive and anti-inflammatory activities (43-45). Therefore, it is possible that the presence of the *E-NPP1* Q/Q genotype has an indirect negative effect on CD39 e CD73 activities, since mutated E-NPP1 will generate less substrate for the other ectonucleotidases of the cascade. This might lead to increased activity of T effectors lymphocytes; thus, predisposing to AR (**Figure 2-B**). To confirm this hypothesis, the functional effect of the Q/Q genoytpe on E-NPP1 activity in kidney transplant patients must be further explored.

It is known that factors that influence immunological sensitization in transplant recipients, such as pregnancy, blood transfusions, and prior transplants, might increase the risk of AR (46). Likewise, donor and recipient characteristics, such as age and ethnicity seem to influence this outcome (47-49). However, HLA compatibility is one of the most important factors leading to AR (50, 51). In our sample, only HLA-DR mismatch, recipient age at transplantation and DGF were associated with AR. Probably due to our sample size, we cannot exclude that the other characteristics discussed above might influence AR in our population. It is noteworthy that the *E-NPP1* K121Q polymorphism remained independently associated with AR after adjustment for HLA-DR mismatch, pregnancy and transfusion numbers, donor and recipient age and DGF.

E-NPP1 K121Q polymorphism is differentially distributed across ethnicities (17, 22). In this context, some studies showed that the Q allele has an increased prevalence among African-Brazilians (27) and other black populations (52-55). Based on this knowledge and also because the vast majority of our sample was comprised of white subjects, we evaluated only white subjects in the present study.

The main limitation of our study is the fact that it is a retrospective study. Some other limitations are: the fact that our Center does not routinely perform HLA-DQ, which is known to frequently induce DSA, and could be related to the risk for AR; and our study represents the experience of one center, and thus, results may not be fully generalized to other settings. Although understanding the need for confirmation in studies with larger samples, we believe that this potentially useful finding should be reported.

In conclusion, our findings support an association between the *E-NPP1* K121Q polymorphism and AR in kidney transplant patients. Screening of this polymorphism may be useful to predict those patients (carriers of Q/Q genotype) more likely to

experience rejection and, therefore, may need a more intense vigilance or even a more intense immunosuppressive therapy. Further studies are needed to confirm and clarify this association.

Acknowledgments

The authors would like to acknowledge the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoa de Nível Superior (CAPES) and Fundo de Incentivo à Pesquisa e Eventos (FIPE) at Hospital de Clínicas de Porto Alegre. L.H.C and D.C are recipients of fellowships from CNPq.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

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Table 1. Demographic and clinical characteristics of kidney transplant recipients classified by presence kidney acute rejection

	Acute KidneyRejection		
	Present (n = 96)*	**Absent (n = 358)***	**P**
Receptor age (years)	42.3 ± 12.7	45.3 ± 12.8	0.038
Donor age (years)	42.6 ± 16.0	42.3 ± 15.1	0.868
Recipient gender (male)	57 (59.4)	217 (60.6)	0.918
Type of donor (deceased)	63 (65.6)	257 (71.8)	0.294
Cold ischemia time (hours)	15.6 ± 10.5	15.5 ± 9.8	0.983
Pregnancy			
0	6 (15.4)	32 (22.7)	0.442
≥ 1	33 (84.6)	109 (77.3)	
Transfusion			
≥ 1	47 (49.0)	163 (47.0)	0.819
Renal replacement therapy			
Hemodialysis	90 (93.8)	326 (91.1)	0.568
Peritoneal dialysis	5 (5.2)	22 (6.1)	
Preemptive transplant	1 (1.0)	10 (2.8)	
Delayed graft function	61 (64.2)	187 (52.2)	0.049
HLA-A MM (0/1/2)	10/48/36	53/173/123	0.521
HLA-B MM (0/1/2)	9/46/39	60/160/129	0.191
HLA-DR MM (0/1/2)	22/43/29	126/156/63	0.009
HLA-A+B+DR MM	3.67 ± 1.5	3.22 ± 1.43	0.008
Hypertension	70 (72.7)	288 (85.2)	0.183
Last PRA Class I (% positive)	33 (37.1)	123 (36.1)	0.958

Last PRA Class II (% positive)	31 (34.8)	101 (29.6)	0.412

Data are presented as mean ± SD, n (%) or n. DSA= donor specific antibody; MM = mismatch; PRA= panel reactive antibody. * Unknown status for transfusion: n = 11, DGF: n = 1, Hypertension: n = 27, Last PRA Class I/II: n = 24.

Table 2. Frequencies of the *E-NPP1* K121Q polymorphism between kidney transplant patients with acute rejection (AR) and without acute rejection (non-AR).

***E-NPP1* K121Q polymorphism**	**AR (n = 96)**	**Non-AR (n = 358)**	**P value**	**P value for additive model[a]**	**P value for dominant model[b]**	**P value for recessive model[c]**
Genotype						
Q/Q	11 (11.5)	11 (3.1)	0.003	0.002	0.278	0.002
K/Q	25 (26.0)	100 (27.9)				
K/K	60 (62.5)	248 (69.0)				
Allele						
Q	0.245	0.170	0.024	-	-	-
K	0.755	0.830				

Data are shown as n (%) or proportion. [a]Q/Q *vs*. K/K; [b]QQ/KQ *vs*. KK; [c]QQ *vs*. KQ/KK. P values were computed by χ^2 tests for comparisons between groups.

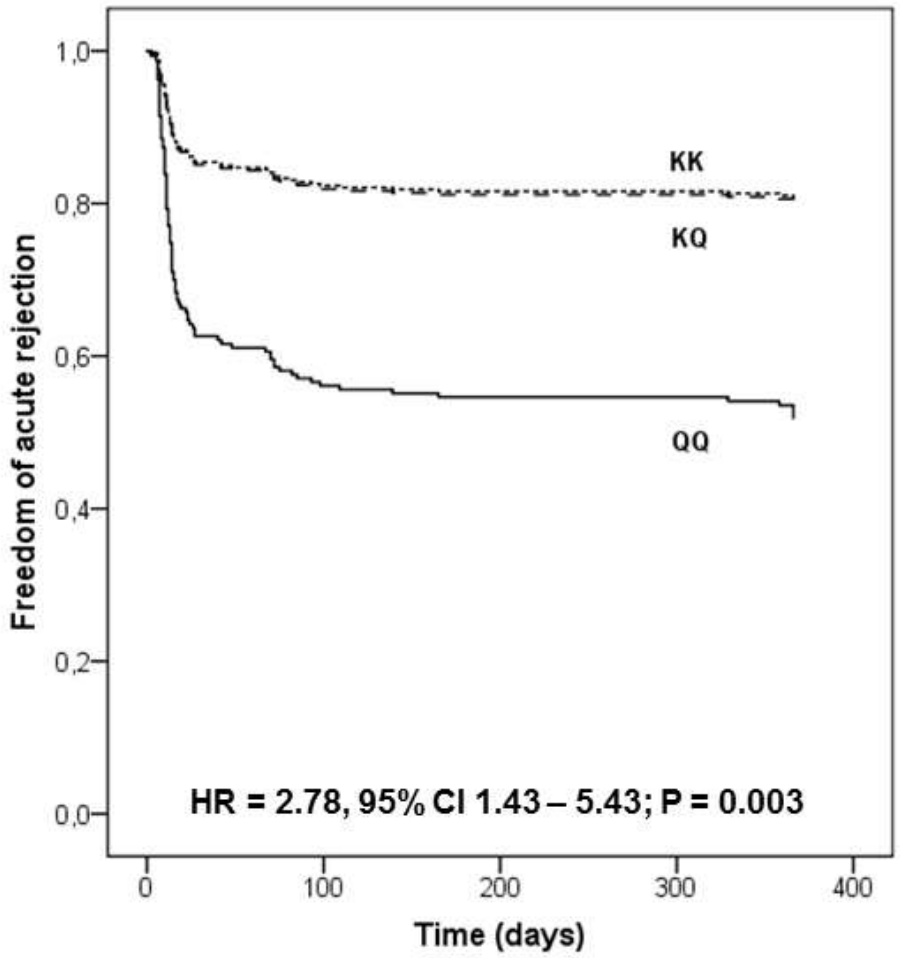


Figure 1. Cox regression analysis of the *E-NPP1* K121Q polymorphism and acute rejection (AR) episodes in kidney transplant recipients. Adjusted for HLA-DR mismatch, pregnancy and transfusion numbers, donor and recipient age and DGF.

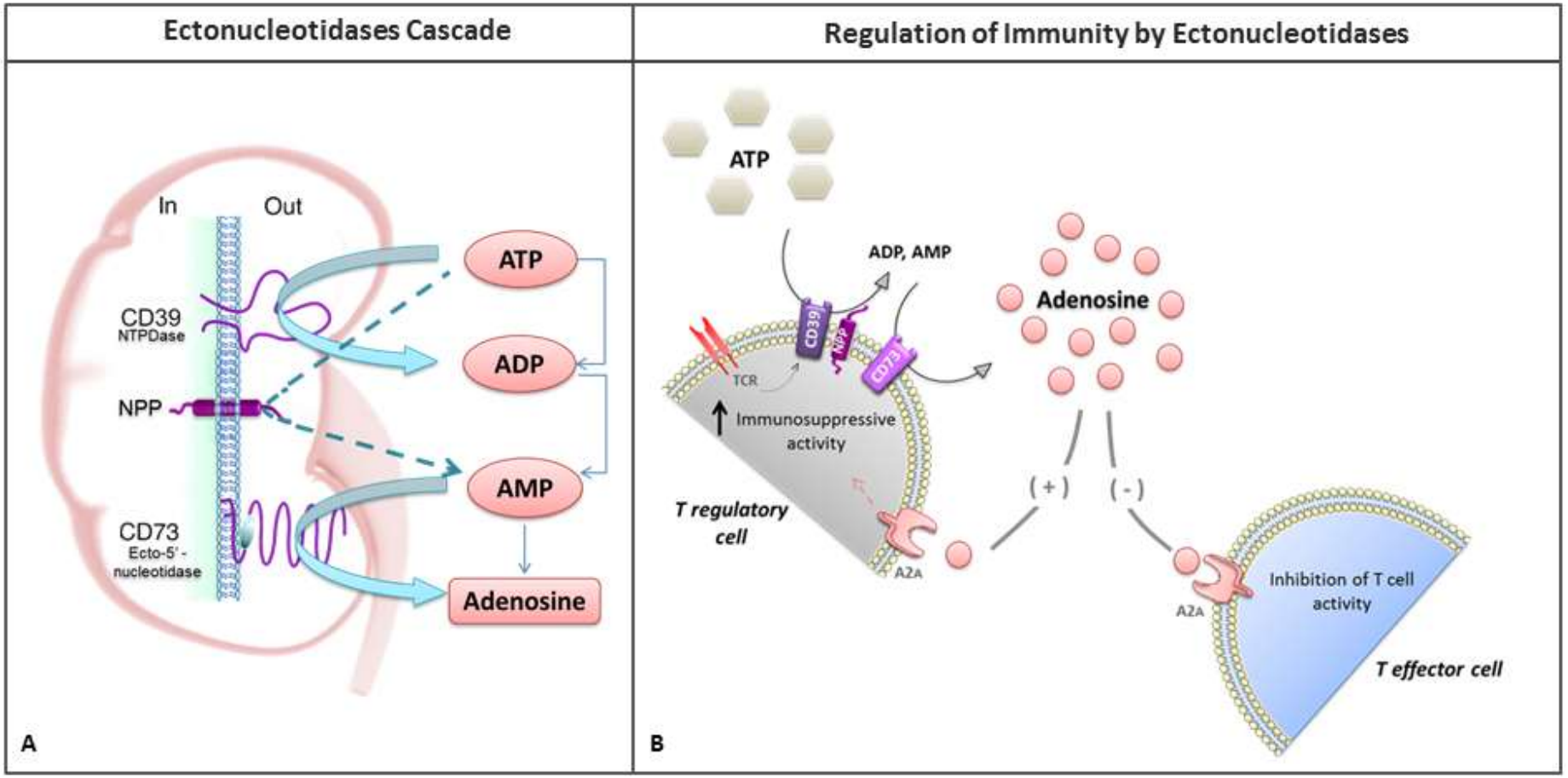
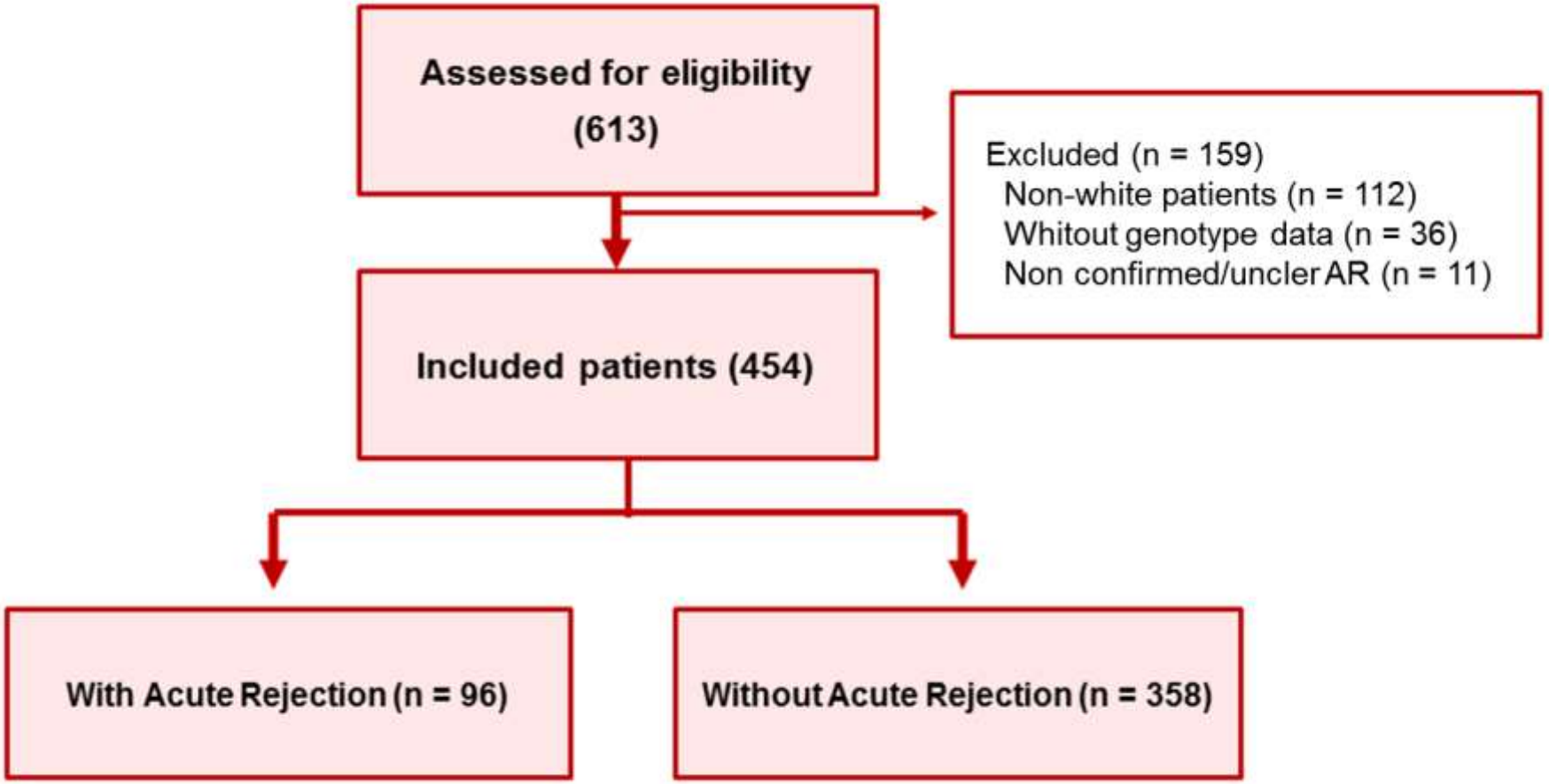

Ectonucleotidases Cascade
Regulation of Immunity by Ectonucleotidases
In
Out
CD39
NTPDase
NPP
CD73
Ecto-5' -
nucleotidase
ATP
ADP
AMP
Adenosine
A
ATP
ADP, AMP
Adenosine
CD39
NPP
CD73
TCR
Immunosuppressive
activity
T regulatory
cell
A2A
(+)
(-)
A2A
Inhibition of T cell
activity
T effector cell
B

Figure 2 - (A) Ectonucleotidases cascade – Members of the NTPDase (CD39) family are cell membrane enzymes that hydrolyze ATP into ADP as well as ADP into AMP through three different steps. In contrast, NPPs are able to degrade ATP and ADP into AMP in a single step, releasing AMP. In the final hydrolyzation step, the extracellular AMP can then be hydrolyzed to adenosine and inorganic phosphate (Pi) by the effect of Ecto-5′-nucleotidase (CD73). **(B) Regulation of immunity by ectonucleotidase cascade –** The occurrence of pathological insults, such as AR, activates T cell receptors (TCR) expressed in T regulatory cells (Treg), which induces CD39/CD73 activity leading to adenosine generation. Increased levels of extracellular adenosine promote immunosuppressive and anti-inflammatory activity in Treg cells. Also, through its receptor A2A in the T effector cell, adenosine suppresses T cell immunity by inhibiting activation of T effector cells. Thus, by employing different mechanisms on Treg and T effector cells, adenosine promotes an immunosuppressive effect.



Supplementary Figure 1: Flowchart showing the strategy used to select patients for inclusion in the study.

6 PERSPECTIVAS

A RA celular é caracterizada essencialmente pela presença de infiltrado intersticial focal ou difuso com predomínio de células mononucleares. Sendo assim, a RA como uma reação imunológica, pode levar a alterações nos componentes celulares sanguíneos, sendo que essas mudanças diversas vezes se refletem em diferentes padrões da expressão gênica durante o desenvolvimento patológico. Alguns estudos demostraram a identificação de linfócitos T citotóxicos em infiltrado mononuclear de rins com rejeição irreversível e a relação destes com expressão de alguns genes. Dado o exposto, o estudo de genes em células com infiltrados mononucleares que possam ser preditores do processo de rejeição é uma estratégia atrativa.

Dessa forma, uma perspectiva do presente trabalho é investigar a expressão do gene *E-NPP1* em células mononucleares de pacientes transplantados renais com e sem RA, bem como avaliar se a expressão do gene *E-NPP1* nessas células é diferente entre os genótipos do polimorfismo K121Q (rs1044498). Os resultados desse estudo contribuirão para o entendimento dos mecanismos pela qual a *E-NPP1* e polimorfismos nesse gene podem contribuir para a patogênese da RA.

7 CONCLUSÃO

Esta tese teve como uns dos objetivos esclarecer a associação do polimorfismo K121Q no gene *E-NPP1* com a DRD através de uma revisão sistemática e metanálise dos estudos disponíveis na literatura. Nossos resultados da metanálise mostraram que o alelo Q do polimorfismo K121Q no gene *E-NPP1* é um fator de risco para DRD considerando diferentes modelos de herança, com uma RC de 1,74 (IC 95% 1,27 – 2,38) para o modelo aditivo.

Também tivemos como objetivo avaliar se este polimorfismo estava associado com RA em pacientes transplantados renais. Os resultados desse segundo estudo demonstraram, pela primeira vez, que o genótipo Q/Q do polimorfismo K121Q foi associado com RA (HR = 2,76, IC 95% 1,44 – 5,30) após ajuste para número de gravidez, transfusões, função tardia do enxerto, idade do doador e número de *mismatch* do HLA.

Apesar do conhecimento adicionado pelos resultados desta tese, mais estudos são necessários para uma melhor compreensão dos mecanismos de ação pelos quais o polimorfismo K121Q no gene *E-NPP1* influencia a DRD em pacientes diabéticos, bem como a RA do transplante renal. Além disso, a fim de confirmar a associação deste polimorfismo com RA, estudos de replicação em outras populações são de suma importância. A confirmação de que este polimorfismo pode ser um biomarcador para risco de RA poderá levar, no futuro, ao desenvolvimento de novas estratégias para proteção do transplante renal.