ISABEL MARIA DA SILVA FONSECA

PREDICTIVE FACTORS OF GRAFT DYSFUNCTION AND LONG-TERM KIDNEY ALLOGRAFT FAILURE

FATORES PREDITIVOS DE DISFUNÇÃO E PERDA DO ENXERTO RENAL A LONGO PRAZO

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientadora – Doutora Denisa Mendonça

Categoria – Professora Associada (Aposentada)

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Coorientadora – Doutora Luísa Lobato

Categoria – Professora Auxiliar Convidada

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Nothing in this world can take the place of persistence.
Talent will not: nothing is more common than unsuccessful men with talent.
Genius will not; unrewarded genius is almost a proverb.
Education will not: the world is full of educated derelicts.
Persistence and determination alone are omnipotent.

Calvin Coolidge

Financial support for this thesis was kindly provided by:

Centro Hospital do Porto

Unit for Multidisciplinary Research in Biomedicine, Porto

Abbott Laboratories (*for providing the kits for measuring urinary NGAL in almost 200 samples*)

Astellas Pharma (*for supporting the acquisition of reagents for measuring oxidative markers)*

Conflict of interest:

The author of this thesis has no conflicts of interest to disclose.

Preface

The work presented in this PhD thesis was conducted at the Department of Nephrology and Kidney Transplantation in close collaboration with the Department of Clinical Chemistry of Centro Hospitalar do Porto, Porto, Portugal.

Because of the difficulties in obtaining funds for the prospective studies, the need for a period of interruption as a result the illness of a close relative, and my concurrent full-time employment, the work included in this PhD thesis was extended and performed from July 2008 to October 2014.

A PhD is a long, complex, meticulous, and, in most cases, laborious and painful process. The prospective cohort-based studies included in this thesis were purposely designed and completed for this PhD project and some of the biomarker analyses were performed for the first time in the Department of Clinical Chemistry. The financial support obtained for this part of the study was exhausted by the purchase of reagents. Thus, I had an active role in accomplishing of this task. Whenever a patient was called for transplant and agreed to participate in the study I was notified and then the study process was triggered. Every day for seven months, including weekends, I prepared and stored approximately 3000 blood samples (whole blood, serum, plasma and erythrocytes). And after that, I actively participated in the laboratory analyses, mainly in the oxidative stress measurements.

This thesis cannot express the long days (and nights!) spent in the lab but it represents a culmination of work, writing and learning. I have been responsible for the design and organization of the study herein, as well as almost all aspects of the data collection and processing. I have learned how to elaborate and conduct research in a complex field, how to collaborate with other researchers as a team and how to conduct research as an individual.

This PhD was a challenging as well as a rewarding journey during which I have gained important knowledge and valuable skills. I have been the main author for all publications. With the exception of the competing risks analysis, which was performed by Laetitia Teixeira, one of my co-authors, all statistical analyses were performed by me (some for the first time) and guided by my main supervisor Denisa Mendonça.

This thesis is the report of this long process. It cannot account the long hours spent on computer with statistics and scientific writing. It cannot express the hope for good results and the sadness and tiredness with each manuscript rejection. But I hope that it expresses hard work, determination and persistence. Do not give up! I believe that this was the hardest lesson on this PhD journey.

Isabel Fonseca,

Porto, November 2014

Acknowledgements

I would like to express my acknowledgement to CENTRO HOSPITAL DO PORTO who provided a research scholarship support for my doctoral study and also to my other funding sources: ABBOTT Laboratories, ASTELLAS Pharma and MULTIDISCIPLINARY UNIT FOR BIOMEDICAL INVESTIGATION, Porto, Portugal. Without these financial supports, this study would not have been possible. Thank you so much!

My acknowledgements are also extended to the PATIENTS who consented to taking part in the studies included in this thesis.

Personnal Thanks

There is a long list of people who have supported, helped and encouraged me throughout my work with this thesis. I wish to emphasize my gratitude to some of you in particular.

My PhD journey has been a process of experiencing, learning and maturing, both in terms of scientific knowledge and personal growth. This thesis would not have been possible without the confidence and motivation of my teacher and main supervisor PROF. DENISA MENDONÇA. She is the main individual responsible for ensuring that I initiated, continued, and completed my research towards my PhD. She did not allow me to give up. I am grateful for her guidance, mentorship, encouragement and friendship. Although I do not say it often, THANK YOU, for being there every day, for giving me the opportunity to learn and work with you, and for exploring the field of scientific research with me. More than anyone else, I owe this thesis to you. THANK YOU SO MUCH FOR EVERYTHING!

I would also like to acknowledge my co-supervisors PROF. LUÍSA LOBATO and DR. ANTÓNIO CASTRO HENRIQUES for their trust, valuable advice and encouraging guidance. Thank you DR. CASTRO for your unconditional support throughout almost 20 years! Thank you LUÍSA for your friendship and for helping me accomplish this achievement especially in these last months. And thank you both for your efforts in achieving funds for this PhD.

I am deeply indebted to LA SALETE MARTINS, MANUELA ALMEIDA, LEONÍDIO DIAS, JOSEFINA SANTOS, SOFIA PEDROSO, and ANTÓNIO CASTRO HENRIQUES for their commitment in patient recruitment and for their invaluable help with many aspects of the work presented in this thesis. Most of all, I would like to gratefully and sincerely thank for your invaluable friendship.

I am also most grateful to DR. ANTÓNIO CABRITA and to all MY NEPHROLOGY CO-WORKERS who, in one way or another, have supported me and without whom I would not have been able to conduct this PhD journey. Special thanks goes to DR. GUILHERME ROCHA and DR. JOÃO PEDRO PIMENTEL for our daily exchange of ideas and thoughts, and for the fun moments together.

I would like to express my appreciation to the NURSES of the Nephrology Department for

collecting the biological samples and for their encouragement and commitment to my doctoral study. I am indebted to you more than you know. Thank you!

I also wish to thank DR. JOSÉ CARLOS OLIVEIRA for his advice on NGAL, adipokines and cystatin C and for allowing me to perform the oxidative stress analyses at his Department. Thank you for allowing me to use the Department's facilities and for trusting me with the Department keys, including on weekends.

I am grateful to HENRIQUE REGUENGO and MARIA LUÍS CARDOSO, my lab instructors, for their friendship, expertise, and technical laboratory support. Thank you both for the incredible patience during my time as a lab assistant and for the long nights in the lab.

I would like to thank to the laboratory staff of the Department of Clinical Chemistry, particularly MADALENA CRUZ, for their precious help, technical assistance and positive attitude over the months that I spent there.

I am also very grateful for the help of PROF. MARTINS DA SILVA for his ongoing support throughout the PhD process and of PROF. PEDRO OLIVEIRA for all valuable comments and advice to improve my thesis.

I appreciate the opportunity and experience of working with LAETITIA TEIXEIRA on an interesting competing risk problem in kidney transplantation. Thank you for your friendship, helpful comments, and encouragements during the last phase of my work.

Thank you JORGE MALHEIRO for your insightful discussions about research, valuable inputs, and help with sampling and methodological issues. Thank you for general breadth of knowledge of all things and for meticulously editing my thesis.

Many friends have encouraged me during the years I have been working with this PhD thesis, and I would like to thank all of them, specially MARGARIDA LIMA, PEDRO AMORIM, LAURINDA LAPA, JOSÉ MANUEL PEREIRA, TERESA MENDONÇA, MARIA MENEZES, PAULA GAMA, JOSÉ QUEIRÓS, ANABELA RODRIGUES, IDALINA BEIRÃO, AND LAURINDA TEIXEIRA.

Special thanks goes to FREDERICO for being my every day computer consultant and for being a source of relaxation through the ups and downs. Thank you for your never-ending support and for giving me happiness. Thank you so much for always being there for me!

Last but not the least, I would like to thank to my best friends, my sisters TITA E SUSANA and my brother Rui, for their incentive and emotional support thoughout this effort. I could not have done it without you! You have helped me more than you know! Thank you, more than words can say.

To MY PARENTS, thank you for your unconditional love, support and encouragement. You two have always inspired me to work harder and pursue my dreams, and for that I will be forever grateful.

Abstract

Kidney transplantation is considered the treatment of choice for many patients with end-stage chronic kidney disease; however, despite advancements in short-term allograft survival, long-term survival has not paralleled this improvement. Due to the inevitable ischemic damage and associated reperfusion injury, delayed graft function (DGF) is a common complication after kidney transplantation, which may negatively affect graft survival. Because serum creatinine (SCr) and other traditional markers of kidney injury are insensitive and delayed in the detection of the early stages of kidney damage and DGF, there has been a keen interest in the identification of novel biomarkers for the early detection of allograft dysfunction that could expedite treatment and improve long-term patient and graft survival. Biomarkers are characteristics that can be objectively measured in a biological sample. In clinical settings, biomarkers enable the diagnosis of a dysfunction or disease and, in some cases, they are used to monitor a treatment or to make a prognosis regarding the future outcome of a patient. The analysis of predictive factors of graft dysfunction and long-term kidney allograft failure focusing on novel biomarkers was the major motivation for this work. Thus, the general aim of this thesis was to investigate the potential of different biomarkers to reliably diagnose and predict early graft dysfunction and their effect on long-term kidney allograft failure as well as to gain insight into the underlying mechanisms of graft dysfunction.

Patients and Methods: The study involved three cohorts of patients: two retrospective cohorts that included kidney transplant recipients selected from a database that contained transplant and follow-up information on kidney transplants performed between 1983 and 2008 (for the first retrospective cohort) or 2012 (for the second retrospective cohort); and one prospective cohort that included 40 patients undergoing kidney transplantation between December 2009 and June 2010. The first retrospective cohort was used to validate the one-year SCr as a surrogate endpoint of long-term graft survival, and the second retrospective cohort was considered to analyze the impact of DGF (defined by the need for dialysis during the first week after kidney transplantation) on graft and patient survival using a competing risks approach. The studies based on the prospective cohort had a longitudinal observational design, which was initiated at the time of transplantation; this cohort was used to examine nine potential candidate biomarkers for the early diagnosis of DGF (one biomarker in urine and eight biomarkers in blood): cystatin C (CysC), neutrophil gelatinase-associated lipocalin (NGAL), leptin and adiponectin, malondialdehyde (MDA), superoxide dismutase (SOD), glutathione reductases (GR), peroxidases (GPx) and total antioxidant status (TAS). Five samples per patient were collected within the first week: 3 to 6 h prior to transplant surgery (pre-transplant); on the

subsequent morning at approximately 8 to 12 h after graft reperfusion (day-1); and then on the second (day-2), fourth (day-4) and seventh (day-7) days after transplant, which resulted in five samples per patient.

A linear mixed effects model was used to evaluate the longitudinal changes of the potential new biomarkers of early graft dysfunction over the first week after kidney transplantation and to identify the factors associated with these changes. The performance of the candidate biomarkers in the prediction of DGF was examined using receiver-operating characteristic (ROC) curves. Survival analysis methods, including a survival analysis that accounted for competing risks were used to identify the predictive factors of long-term graft survival.

Results: Of the large number of variables that were considered, the SCr levels at 1, 6 and 12 months following kidney transplantation, as well as the changes between 1 and 6 months and between 6 and 12 months were independently associated with late graft failure.

The ROC curves identified urinary NGAL, MDA and CysC on the first postoperative day as moderately (NGAL) and highly (MDA and CysC) accurate in the prediction of DGF. Both urinary NGAL (at days 4 and 7) and MDA (day-7) were independently associated with one-year graft function, adjusting for variables that typically affect graft function, including acute rejection episodes and re-admissions during the first post-transplant year.

Leptin at day-1 was slightly better than SCr in the prediction of the need for dialysis within the first week post-transplant, whereas adiponectin, SOD, GR, GPx and TAS were not. A triple-biomarker approach that used SCr, CysC, and MDA measured 8 to 12 h after kidney transplantation, was the most informative combination, which resulted in an increased ability (AUC=0.96) to distinguish patients with graft damage who would require dialysis within the first week. The application of a subdistribution regression model for competing risks indicated that DGF by itself and independent of acute rejection had a detrimental effect on long-term graft survival, but not on patient survival.

Conclusions: Independent of acute rejection, DGF *per se* was significantly associated with poor-graft survival, but not with patient survival. Urinary NGAL and serum CysC and MDA were early, noninvasive, and accurate predictors of both the need for dialysis within the first week of kidney transplantation and one-year graft function. A triple-biomarker approach using SCr, CysC and MDA were highly predictive of DGF. Combining biomarkers from different pathophysiologic pathways appears to be a rational and reliable strategy to optimize sensitivity and specificity and obtain additive diagnostic and prognostic information.

Resumo

O transplante renal (TR) é considerado o melhor tratamento para a maioria dos doentes com necessidade de substituição da função renal. Apesar dos progressos alcançados, principalmente a nível da falência do enxerto nos primeiros seis meses após TR, a sobrevivência a longo prazo não tem acompanhado essa evolução. A ocorrência de atraso de função do enxerto (AFE), nomeadamente por lesão provocada pela isquemia e reperfusão associada ao transplante, condiciona a evolução do pós-transplante e tem um impacto negativo nos resultados imediatos e a longo prazo do TR. O desenvolvimento de intervenções eficazes na prevenção e/ou atenuação da agressão precoce no enxerto renal tem sido limitado pela ausência de marcadores precoces da lesão e disfunção renal. Os biomarcadores são substâncias ou "entidades" objetivamente quantificáveis, indicadores do curso de um processo biológico normal ou da ocorrência de uma lesão ou processo patológico, sendo usados na prática clínica para diagnóstico, monitorização terapêutica, estratificação de risco e previsão de eventos. Tendo em conta que os marcadores tradicionais de lesão e função renal, como a creatinina sérica (SCr), são tardios e insensíveis para o diagnóstico atempado de AFE, têm sido procurados novos biomarcadores capazes de identificar precocemente a disfunção renal e promover uma intervenção atempada e uma melhoria da sobrevivência a longo-prazo do enxerto renal. A análise dos fatores preditivos de disfunção e perda do enxerto renal a longo prazo, com ênfase na investigação de potenciais biomarcadores da disfunção precoce do enxerto, expressa pelo AFE, e do seu efeito na sobrevivência renal a longo prazo foi a principal motivação e objetivo desta tese.

Participantes e Métodos: O estudo envolveu três coortes: duas retrospetivas em que os participantes foram selecionados a partir da base de dados do TR da Unidade de Nefrologia e Transplante Renal do Centro Hospitalar do Porto de 1983 a 2008 (para a primeira coorte retrospetiva) ou 2012 (para a segunda coorte retrospetiva) e uma coorte prospetiva de 40 doentes convocados para TR entre Dezembro de 2009 e Junho de 2010. A primeira coorte retrospetiva foi usada para validar a SCr observada durante o primeiro ano pós-TR como um marcador *surrogate* (substituto) da sobrevivência do enxerto renal a longo-prazo. A segunda coorte retrospetiva foi utilizada para avaliar o impacto do AFE (definida pela necessidade de diálise na primeira semana pós-TR) na sobrevivência do doente e do enxerto renal a longo prazo usando uma abordagem estatística baseada em eventos competitivos. Os estudos baseados na coorte prospetiva seguiram um desenho observacional longitudinal com início à data do transplante e pretenderam estudar nove potenciais biomarcadores para o diagnóstico precoce de AFE (um na urina e oito no sangue): a cistatina C (CysC), a lipocalina associada a gelatinase

dos neutrofilos (NGAL), alguns marcadores de stress oxidativo [malondialdeído (MDA), glutationa peroxidase (GPx) e reductase (GR), superóxido dismutase (SOD) e a capacidade antioxidante total (TAS)] e as adipocinas leptina e adiponectina. Foram colhidas 5 amostras por doente durante a primeira semana pós-TR: 3 a 6h antes do transplante; na manhã subsequente, aproximadamente 8 a 12 h após a reperfusão do enxerto renal (dia-1); e depois no segundo, quarto e sétimo dias pós-TR. A evolução longitudinal dos valores dos marcadores durante a primeira semana pós-TR e a identificação de fatores associados às alterações analíticas observadas nessa semana foram estudadas por modelos lineares de efeitos mistos. O estudo da *performance* dos biomarcadores no prognóstico de AFE foi efetuado com as curvas ROC. Métodos de análise de sobrevivência, incluindo a componente de eventos competitivos, foram usados para identificar fatores preditivos da sobrevivência do enxerto renal.

Resultados: Ajustando para os fatores tradicionalmente associados à perda a longo prazo do enxerto renal, a SCr aos 1, 6 e 12 meses, assim como a diferença entre os valores de SCr entre primeiro e o sexto mês e entre o sexto e o primeiro ano associaram-se de forma significativa e independente à perda de enxerto renal a longo-prazo. As curvas ROC revelaram que o NGAL urinário, o MDA e a CysC séricos no primeiro dia pós-TR foram moderadamente (NGAL) e fortemente (MDA e CysC) mais sensíveis no diagnóstico de AFE. Tanto o NGAL urinário (aos dias 4 e 7), como o MDA (ao dia-7) se associaram de forma independente à função renal observada no primeiro ano pós-TR, ajustando para os fatores que tradicionalmente afetam a função do enxerto. Os valores de leptina no primeiro dia pós-TR apresentaram uma *performance* ligeiramente melhor que a SCr para predizer o AFE, o que não ocorreu com a adiponectina, SOD, GR, GPx e TAS. Um multimarcador composto por SCr, MDA e CysC resultou da combinação de marcadores com melhor capacidade preditiva 8 a 12h após o TR (AUC=0.96) para identificar os doentes com lesão do enxerto renal e predizer a necessidade de diálise durante a primeira semana pós-TR. A aplicação de modelos de regressão de subdistribuição para eventos competitivos permitiu demonstrar que o AFE isolado e independentemente da rejeição aguda tem um efeito deletério na sobrevivência do enxerto renal, mas não na sobrevivência do doente.

Conclusões: O AFE por si só e independentemente da rejeição aguda associou-se a pior sobrevivência do enxerto renal, mas não do doente. O NGAL urinário, o MDA e a CysC séricos são marcadores precoces e preditores da necessidade de diálise durante a primeira semana pós-TR e da função renal ao primeiro ano. Um marcador composto triplo com SCr, CysC e MDA foi altamente preditivo de AFE. A combinação de marcadores procedentes de diferentes vias patofisiológicas é uma estratégia racional para optimizar a sensibilidade e a especificidade e obter informação diagnóstica e prognóstica adicional.

List of Publications included in the PhD Thesis

ORIGINAL ARTICLES

I. **Fonseca I**, Almeida M, Martins LS, Santos J, Dias L, Lobato L, Henriques AC, Mendonça D. First year renal allograft function predicts long-term renal allograft loss. *Transplant Proc*. 2011; 43: 106-12

II. **Fonseca I**, Oliveira JC, Almeida M, Cruz M, Malho A, Martins LS, Dias L, Pedroso S, Santos J, Lobato L, Henriques AC, Mendonça D. Neutrophil Gelatinase-Associated Lipocalin in kidney transplantation is an early marker of graft dysfunction and is associated with one-year renal function. *J Transplant*. 2013; 2013: 650123. doi: 10.1155/2013/650123. Epub 2013 Oct 31

III. **Fonseca I**, Reguengo H, Almeida M, Dias L, Martins LS, Pedroso S, Santos J, Lobato L, Henriques AC, Mendonça D. Oxidative stress in kidney transplantation: Malondialdehyde is an early predictive marker of graft dysfunction. *Transplantation* 2014; 97: 1058-65.

IV. **Fonseca I**, Oliveira JC, Santos J, Martins LS, Almeida M, Dias L, Pedroso S, Lobato L, Henriques AC, Mendonça D. Leptin and Adiponectin during the first week after kidney transplantation: Biomarkers of graft dysfunction?

(*In Press, Accepted Manuscript in Metabolism – Clinical and Experimental)*

V. **Fonseca I**, Reguengo H, Oliveira JC, Martins LS, Malheiro J, Almeida M, Santos J, Dias L, Pedroso S, Lobato L, Henriques AC, Mendonça D. A Triple-Biomarker approach for the detection of delayed graft function using serum creatinine, cystatin C, and malondialdehyde. (*Submitted)*

VI. **Fonseca I**, Teixeira L, Malheiro J, Martins LS, Dias L, Henriques AC, Mendonça D. The Effect of delayed graft function on graft and patient survival in kidney transplantation: An approach using competing events analysis. *Transplant International* 2015; 28: 738-50.

REVIEW ARTICLE

VII. **Fonseca. I.** Biomarkers in kidney transplantation: Translating to clinical practice (Review Article). *Port J Nephrol Hypert* 2013; 27: 143-151

Table of Contents

Keywords

Biomarkers
Kidney transplantation
Delayed graft function
Long-term graft failure
Cystatin C
Leptin
Neutrophil gelatinase-associated lipocalin
Oxidative stress
Malondialdehyde

List of Abbreviations

ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
AUC	Area under the curve
ATP	Adenosine triphosphate
CIF	Cumulative incidence function
CKD-EPI	Chronic kidney disease epidemiology collaboration
csHR	Cause-specific hazard ratio
CysC	Cystatin C
DGF	Delayed graft failure
eGFR	Estimated glomerular filtration rate
EDTA	Ethylenediaminetetraacetic acid
GFR	Glomerular filtration rate
GPx	Glutathione peroxidase
GLM	Generalized linear models
GR	Glutathione reductase
HLA	Human leukocyte antigen
H_2O_2	Hydrogen peroxide
KIM-1	Kidney injury molecule 1
KM	Kaplan-Meier method
1-KM	The complement of Kaplan-Meier estimator/estimates
MANOVA	Multivariate analysis of variance
MDA	Malondialdehyde
MnSOD	Manganese superoxide dismutase
NGAL	Neutrophil gelatinase-associated lipocalin
$O_2^{-\cdot}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
sHR	Subdistribution hazard ratio
SCr	Serum creatinine
SOD	Superoxide dismutase
TAS	Total antioxidant status
uNGAL	Urinary neutrophil gelatinase-associated lipocalin

Chapter 1

General Introduction

Content

Background and motivation for the work in this thesis
Aims and objectives
Thesis outline

BACKGROUND AND MOTIVATION FOR THE WORK IN THIS THESIS

Kidney transplantation is considered the treatment of choice for almost all cases of renal failure, particularly because the quality of life and patient survival associated with transplantation are better than for chronic dialysis.[1] Due to the new immunosuppressive drugs and consequent decrease in the rejection incidence, the short-term outcome of renal transplantation has improved substantially in the past 20 years. However, despite progress in short-term allograft survival, long-term survival has not paralleled that improvement.[2-6]

At present, late failure of kidney transplants is an important clinical problem and one of the leading causes of end-stage renal disease.[7] The rate of chronic graft loss after the first year remains significant and the actual kidney allograft half-life showed only a marginal improvement over the past decade.[2, 3, 6, 8] The reasons for this slight improvement remain unclear. It is possible that some important determinants of long-term graft survival may not have changed sufficiently to improve the overall outcomes of kidney transplantation.[9] Patient death with a functioning allograft, mostly from cardiovascular disease, and chronic allograft failure are the two major causes of late transplant loss.[10-12] The causes of chronic allograft failure are multifactorial and are influenced by numerous immunological and non-immunological factors.[2, 9] Generally, kidney transplants stabilize after recovering from the stress of implantation until declining of graft function due to specific diseases or conditions, such as recurrent renal disease, antibody-mediated rejection or a common process involving interstitial fibrosis and tubular atrophy, which is encompassed by the previous descriptive term “chronic allograft nephropathy” and, more recently, “fibrosis / atrophy”.[9, 13-15]

Approximately half of deceased renal allografts are lost within 10 to 12 years after transplantation.[16, 17] A patient submitted to kidney transplantation would wonder whether his or her transplanted kidney will work well and how long it will last. There are no answers to these questions. Clinicians lack appropriate non-invasive methods to predict, diagnose and reduce the risk of graft failure in the years following renal transplantation.

When will it be possible to identify valuable markers for distinguishing patients who are at an increased risk of graft dysfunction or of losing their transplant? Can biomarkers signal early transplant dysfunction, a process that is often undetectable? Can biomarkers help clinicians fine-tune their prognoses?

Many donor, recipient and immunologic characteristics are consistently associated with poor long-term outcomes, namely female gender, black ethnicity, prolonged pre-transplant dialysis time, older donor age, deceased donor source, delayed graft function (DGF), and acute rejection.[11, 18-21] The association of human leukocyte antigen (HLA) matching and panel reactive antibodies with the change in graft function suggests that immune mechanisms continue to have an effect on allograft function even among the long-term transplant recipients.[22] Many of these factors coexist and act synergistically, and DGF is one of them.

Delayed graft function is a well-known and the most common complication in the immediate post-transplantation period mainly in deceased renal allografts, almost invariably in the non-heart beating and in some live donor transplants.[23-25] This condition is a continuous spectrum of ischemia-reperfusion-related acute kidney injury and describes dysfunction of the kidney allograft immediately after transplantation.[23, 26] Although not confirmed by some,[27, 28] most studies have found associations between DGF and an increased risk for acute rejection and chronic allograft dysfunction,[11, 29, 30] worse graft survival[21, 30-35] and higher mortality.[26, 36-38]
A range of factors could lead to DGF such as organ procurement (i.e. kidneys from non-heart-beating donors), donor characteristics (i.e. donors older than 55 years, donors with diabetes and/or high blood pressure), prolonged ischemia time, recipient factors (such as male gender, longer waiting time on dialysis, number of recipient's previous transplants), renal toxicity, and ureteral obstruction, among others.[39] With the present disparity between supply and demand for organs, transplantation is proceeding with more marginal kidneys and therefore the problem of DGF is likely to increase in the future. Thus, DGF poses a significant challenge to clinicians in the context of kidney transplantation.

Ischemia/reperfusion injury after organ transplantation is a major cause of DGF, which is associated with prolonged hospital stay, additional invasive procedures, supplementary costs and greater risk of early and long-term graft loss.[40] The association between DGF and worse outcomes has led to increased efforts to better understand the mechanisms of ischemia-reperfusion injury and to develop interventions to reduce its occurrence and impact. This has included initiatives to discover and use biomarkers to stratify the risk of DGF, to diagnose dysfunction early and to target any intervention to those patients who will benefit most. This was the primary motivation for this thesis.

Thus, the major portion of this work focuses on one critical event after transplantation that is detrimental to the long-term graft and patient survival, DGF, as an expression of acute graft dysfunction. The following are some of the questions that this work tried to answer:

a) Are there biomarkers that are significantly different between patients undergoing DGF, *versus* those who are not?

b) Can a specific combination or panel of biomarkers work together and be potentially utilized for the diagnosis of DGF?

c) What can the identified biomarkers tell us about the underlying pathophysiology of this condition?

d) What is the impact of DGF on the patient and long-term graft survival over decades when using a competing risks approach?

AIMS OF THIS DISSERTATION THESIS

The analysis of predictive factors of graft dysfunction and long-term kidney allograft failure with a main focus on novel biomarkers was the major motivation for this work.

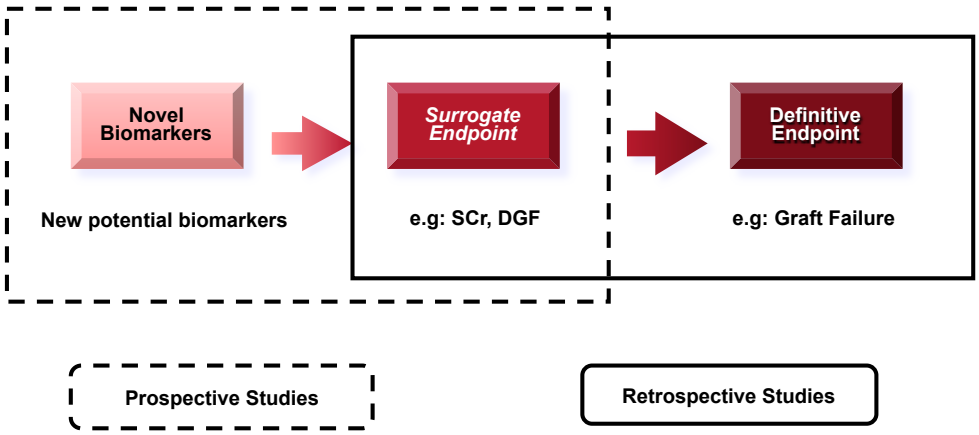
Thus, the general aim of this thesis was to investigate the potential of different biomarkers to reliably diagnose and predict early graft dysfunction and their effect on long-term kidney allograft failure as well as to gain insight into the underlying mechanisms of graft dysfunction.

The evaluation of the role of novel biomarkers on long-term graft failure requires a prospective approach that is impossible to achieve in a short-time period. On a short-term approach, alternative endpoints or short-term markers that can predict graft failure in the long-term may represent potentially useful surrogates and can be used in place of conventional endpoints.

Thus, a phased approach was required to evaluate the clinical utility of novel biomarkers on long-term outcome, with the following design (Fig. 1) and specific aims:

- Develop a surrogate marker for long-term graft survival and evaluate the impact of early graft dysfunction, expressed by DGF, on the long-term graft loss and patient survival. (*Retrospective studies*)

- Identify novel diagnostic biomarkers for early graft dysfunction by investigating the prognostic performance of the candidate biomarkers in the prediction of DGF and one-year graft function. (*Prospective studies*)



DGF: delayed graft function; SCr: serum creatinine

Fig. 1: Research design of this PhD thesis

THESIS OUTLINE

The present thesis is divided into six chapters.

Chapters 1 and 2 contain the scientific background and motivation for the research performed in this thesis with respect to the investigation of novel graft dysfunction biomarkers.

Chapter 1 is a general introduction to some of the achievements and problems of kidney transplantation and summarizes the main objectives and outline of the thesis.

Chapter 2 is as an introductory chapter to the field of biomarkers with relevant definitions and emphasizes the importance of novel and early markers of graft dysfunction in the immediate post-transplantation period, focusing on the biomarkers investigated in the current study.

The materials and methods are described in the original publications (I to VI), but some additional and more detailed information about the procedures and methodology are presented in **Chapter 3**.

Chapter 4 presents an overview of the results and the original research papers included in this thesis as well as a review article. All published papers are reproduced with permission from the publisher.

Paper I aimed to develop a surrogate marker for long-term graft survival in our center. Thus, factors associated with late kidney graft failure were identified and the predictive effect of serum creatinine (SCr) within the first year on long-term graft survival was examined.

In **Papers II, III and IV** the aims were to evaluate longitudinal changes of potential new biomarkers of early graft dysfunction over the first week after kidney transplantation and identify factors associated with these changes, to assess the performance of these candidate biomarkers in predicting DGF, and to appraise the long-term prognostic value of these biomarkers on kidney allograft function, evaluated by one-year SCr. These papers addressed the objectives of the prospective component of this work.

Paper V aimed to combine the studied biomarkers and develop a high sensitive approach to diagnose DGF.

The aim of **Paper VI** was to evaluate the controversial impact of DGF on long-term graft loss and patient survival using a competing events approach.

Paper VII is an invited review article that presents a general discussion about biomarkers in kidney transplantation, integrating some of the findings and biomarkers studied in this thesis.

Chapter 5 provides a general discussion of the main findings of the papers included in this thesis and also considers aspects and reflections that were not included in the papers.

Chapter 6 provides a conclusion to the thesis and presents future perspectives.

Chapter 2

Literature Review

Content

BIOMARKERS

A number of factors concerning donor, recipient and the peritransplant period influence the long-term graft outcome and have been widely discussed in the published literature.[41-46] Although the synergic action of immune and non-immune factors cannot be forgotten, this literature review chapter will focus on biomarkers dependent of peritransplant kidney injury processes.

WHAT IS A BIOMARKER?

Biomarker is a broad term that can be used to describe any indicator of a biological state. The term biomarker, or biological marker, was introduced in 1989 as a Medical Subject Heading (MeSH) term and it was defined as "*measurable and quantifiable biological parameters (e.g., specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances), which serve as indices for health- and physiology-related assessments*." More recently, in 2001, the definition was standardized by the Biomarker Definitions Working Group[47, 48] as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.*"

These definitions correspond to two concepts: first, the objective measurement of a parameter in a biological sample, and second, its application to classifying a patient. Although the term "biomarker" is relatively new, biomarkers have been used in preclinical research and clinical diagnosis for some considerable time. Body temperature is a long-standing and well-known biomarker for fever, for example.

Several types of objective biomarker measurements can be performed on patients. In fact, biomarkers appear in every form. They can be anatomic, physiologic, biochemical, or molecular parameters that are associated with the presence and severity of specific diseases and they are detectable by a variety of methods including physical examination, laboratory assays, and imaging. Serum creatinine (SCr) and blood glucose levels are biomarkers, as are blood pressure, enzyme levels, tumor size measurements obtained by imaging techniques, and the presence of a gene mutation or the expression level of mRNA. Unlike what is commonly believed, biomarkers are not only molecules. A biomarker is any type of measurable change that may have clinical relevance.[49, 50] And this list is far from exhaustive and countless measurements have been proposed as biomarkers.

USEFULNESS AND ADVANTAGES OF BIOMARKERS

The goal of a biomarker measurement is to make a useful prediction about the classification of the patient. In practice, biological markers are used to determine the disease status, monitor the efficacy of a treatment or predict the future outcome of a patient. In any case, using the biomarker values, one would like to split the patients into two classes according to the status of interest. Patients are typically labeled according to a known test that was assessed with certainty, called a gold standard. After identifying specific and accurate biomarkers, future patients will be classified without the need for the gold standard, usually more expensive and risky. Biomarkers are often cheaper and easier to measure than "true" endpoints.

Biomarkers usually provide information that is readily available and simple to interpret by clinicians.[51] For example, a patient's blood pressure is easier to use than echocardiography for measuring left ventricular function, and it is much easier to perform echocardiography than to measure morbidity and mortality from hypertension in the long term. Biomarkers can also be measured more quickly and earlier. Blood pressure can be measured today, whereas it takes several years to collect mortality data.

The usefulness of biomarkers is highlighted by the wide array of clinical settings, in which they are utilized, including disease diagnosis and prognosis. According to the Food and Drug Administration and regardless of the purpose for its use, biomarkers should be accurate, reproducible and standardized across different clinical settings. Ideally, a biomarker should be specific, sensitive, predictive, robust, simple, accurate, and inexpensive. In other words, it should be perfect and improve our understanding of a disease while providing new knowledge of pathological mechanisms, allowing for earlier diagnosis and the delivery of more efficacious and safer therapies possible.[52, 53]

CLASSIFICATION OF BIOMARKERS

Presently, it is not well established how biomarkers are categorized because they can be classified based on different parameters. Within the field of health care, biological markers are commonly classified based on the sequence of events from exposure to disease, including *biomarkers of exposure*, which are used in risk prediction, and *biomarkers of disease*, which are used in screening, diagnosis and prognosis.[51] Clinically, biomarkers may be distinguished according to their uses; an early intervention biomarker is used for the early detection of disease to facilitate intervention, whereas a prognostic biomarker is

correlated with outcomes and is used to identify patients who may benefit from an intervention.[54]

The Biomarker Working Group further classified biomarkers based on their utility and this categorization is also commonly used in biomedical research:[47, 48, 55]

- ***Type 0 biomarker:*** A marker of the natural history of a disease that correlates longitudinally with known clinical indices such as symptoms over the full range of disease states;
- ***Type I biomarker:*** A marker that usually determines the biological effect of a therapeutic intervention according to the mechanism of action of that intervention (pharmacological, nutritional or any other), even though the mechanism might not be known to be associated with the clinical outcome.
- ***Clinical endpoint:*** An outcome that represents the target measures of a study. A characteristic or variable that reflects how a patient fares or functions, or how long a patient survives. In renal transplantation, for example, the standard clinical endpoints are graft failure and death for late outcomes.
- ***Surrogate endpoint biomarker (Type II biomarker):*** A marker that is intended to substitute a clinical endpoint. A surrogate endpoint is expected to predict the clinical benefit, harm, lack of benefit, or lack of harm on the basis of epidemiologic, therapeutic, pathophysiologic, or other scientific evidence. It is important to note that all surrogates are predictors, but not all predictors are surrogates.

Biomarkers versus Surrogates

It is important to distinguish, at the outset, the use of the term biomarker from that of surrogate endpoint or surrogate marker. The use of the term 'surrogate marker' in medicine dates from 1988,[56] but it was preceded for some years by the term 'biomarker'[57] and was succeeded and replaced by yet another term, 'surrogate endpoint'.[58] Surrogate endpoints may be a subset of biomarkers. Although all surrogate endpoints may be considered biomarkers, it is clear that only a few biomarkers will meet the requirements for achieving inclusion in this subset.

A surrogate endpoint is one that is measured in place of the biologically definitive or clinically meaningful endpoint, and it usually tracks the progress or extent of the disease. Investigators choose a surrogate endpoint when the definitive endpoint is difficult to obtain or inaccessible due to cost, time, or the complexity of measurement. As explained by Lachenbruch[54] "a 'surrogate' variable is one that is used in lieu of the true endpoint, to evaluate the outcome more rapidly, less expensively, and/or less invasively." Some examples include CD4 counts in AIDS patients, tumor size reduction in cancer patients,

blood pressure in cardiovascular disease, intraocular pressure in glaucoma patients, and SCr in chronic kidney disease.

Since approximately 1989, biostatisticians have investigated approaches to evaluating whether a biological parameter that might serve as a substitute or "surrogate" for a clinical endpoint in the study of a particular therapy for a particular disease.[48] A "perfect" surrogate endpoint, as described by Prentice,[59] can be measured simply and without invasive procedures, is related to the causal pathway for the definitive endpoint, yields the same statistical inference as the definitive endpoint, and should be responsive to the effects of treatments. The disease affects the surrogate endpoint, which in turn affects the definitive endpoints. This is more than a correlation between the surrogate and the true clinical point. For example, to accept a classification scheme for a biopsy score as a surrogate endpoint for graft survival requires that the biopsy score not only correlates with outcome but that changes in the outcome due to treatment or any other intervention are reflected in the biopsies.[54] Accepting a biomarker as a surrogate for a clinically definitive endpoint requires validation. To validate an endpoint as a legitimate surrogate endpoint, a meta-analysis is usually required because relationships presented in one study may not be generalizable to another. Then, for use in clinical practice, each center should test whether that surrogate works well in the local scenario because populations have different characteristics and therapeutic interventions and treatments are different across countries and centers.

SURROGATES AND BIOMARKERS IN KIDNEY TRANSPLANTATION

One of the concerns in transplant research is to obtain insight into the factors that are associated with long-term allograft survival and to identify early markers of chronic allograft dysfunction, as well as potential interventional pathways. Long-term graft survival is an ideal endpoint, but evaluating an outcome in the long term is usually difficult and time-consuming. For this reason, an easier approach is to identify alternative endpoints or short-term markers that can predict the long-term survival and therefore act as potentially useful surrogates. This approach is widely used in clinical research on cancer and cardiovascular disease and has recently been applied in the context of renal disease.[60]

Biomarkers used for screening or diagnosis also often represent surrogate manifestations of the disease or dysfunction. This is the case for oxidative stress markers in the process of renal ischemia-reperfusion injury following kidney transplantation. Both biomarkers and surrogates significantly contribute to early diagnosis, longitudinal prognoses, and outcome prediction. They often enable the detection of renal graft dysfunction when kidney injury is subclinical, allowing for faster evaluation of drug therapies, transplant techniques, and

patient care protocols. As a result, there has been a concerted effort within the transplant community to attain a diagnostic marker that may serve as a surrogate for eventual graft loss.

Standard biomarkers for kidney damage

Traditional non-invasive markers of kidney injury are insensitive and nonspecific in the detection of early stages of kidney injury. Standard biomarkers for kidney damage include the glomerular filtration rate (GFR), SCr and urea as well as several urine qualities such as proteinuria and hematuria. For decades, the increase in SCr has been the only detectable sign of a reduction in the GFR. At present, a decline in the SCr is still the traditional marker for detecting graft functional recovery after transplantation. However, this biomarker is an unreliable indicator of kidney function during an episode of acute injury.[61] Serum creatinine changes are not specific for parenchymal damage and occur long after the event. It is estimated that more than 50% of kidney function is lost before the SCr rises, which makes SCr less sensitive to early kidney damage and the severity of dysfunction.[61-63] As such, renewed efforts to improve long-term survival through enhanced monitoring and diagnosis of short and long-term graft dysfunction have directed attention to the search of better biomarkers.

Why do we need new biomarkers for kidney transplantation?

In organ transplantation, initial graft dysfunction is one of the most important early postoperative problems, which is mainly due to the unavoidable ischemia-reperfusion injury that occurs in the transplanted organ. In kidney transplantation, ischemic injury of the renal allograft is a critical early insult that augments the risk of acute tubular necrosis and long-term graft loss.[64, 65] The development of effective interventions is constricted by the limited ability to detect graft dysfunction early. The delay period between initiation of injury and clinical and biochemical detection of renal damage calls for the use of more reliable and earlier markers of kidney graft damage. As previously stated, current clinical indicators of kidney injury, such as SCr, are inadequate for timely diagnosis and prognosis. Thus, the application of biomarkers in the field of kidney transplantation will allow for the detection of incipient graft dysfunction, refine diagnoses and enable more effective post-transplant management, potentially improving the short-term (e.g., delayed graft function, acute rejection) and long-term (e.g., allograft failure) outcomes.

LOOKING FOR NEW BIOMARKERS OF KIDNEY GRAFT DYSFUNCTION

The search for new biomarkers is expanding at an unprecedented rate. Recent efforts to identify biomarkers in kidney transplantation with early diagnostic and prognostic potential have yielded several candidates, including neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), cystatin C, interleukin-18, clusterin, kariopherins, glutathione S-transferase iso-enzymes, liver-type fatty acid binding protein, alpha-1-microglobulin, C-terminal agrin fragment and haptoglobin.[66-77] Nevertheless, due to the lack of evidence to support their use in routine, neither of these is currently used in clinical practice. And the search for an ideal marker continues.

In this dissertation, nine candidate biomarkers were studied and they are briefly reviewed in the following paragraphs.

CYSTATIN C

Cystatin C (CysC) is a monomeric, non-glycosylated polypeptide chain of 120 aminoacids with a low molecular mass of 13.3 kDa. This cystatin is produced at a constant rate by nearly all-human nucleated cells and it can be found in virtually all tissues and bodily fluids, preferentially in the cerebrospinal fluid, seminal plasma, and milk.[78, 79] Its concentration is the highest of all known low molecular weight cystatins in most of the extracellular fluids in humans at approximately 1–10 mg/L.[78] Cystatin C is an endogenous cysteine proteinase inhibitor belonging to the type 2 cystatin superfamily. Cysteine proteases are enzymes that are responsible for many crucial physiological processes, such as intracellular protein degradation, apoptosis, major histocompatibility complex class II immune responses, prohormone processing and bone remodeling.[80] By inhibiting the function of several cysteine proteases, CysC participates in the regulation of the balance of catabolism and modulates many of these normal body processes.[81] Other functions for CysC include a role in the atherosclerotic process,[82] antigen presentation,[83] defense against bacteria and viruses[84] and as a growth factor for neural stem cells.[85]

The main catabolic site of CysC is the kidney; more than 99% of the protein is cleared from the bloodstream by glomerular ultrafiltration and tubular reabsorption**.** Because it is not secreted by the tubules, its concentration in urine in normal states is remarkably lower and approximately 0.1 mg/L (Lofberg and Grubb, 1979; Poulik et al., 1983). As CysC is *per se* produced at a constant level, its concentration in the circulation remains nearly stable when kidney function is normal.[79, 86] Consequently, the rate at which CysC C is filtered at the glomerulus is the primary determinant of the blood CysC level. Due to these properties, CysC was first suggested as a marker of the glomerular filtration rate (GFR) in

1985.[87] Since then, CysC has been extensively investigated in multiple clinical studies on adults, children, and in the elderly. In almost all clinical studies, CysC demonstrated a better diagnostic accuracy than SCr in discriminating normal from impaired kidney function.[88, 89]

In fact, CysC fulfills many criteria that are set for an ideal endogenous biomarker of kidney function. As a low molecular weight protein, it is almost freely filtered through the glomerular membrane and then completely reabsorbed and catabolized by the proximal tubular cells.[86, 90, 91] In studies performed with mice, the plasma clearance of CysC is 94% that of 51Cr-EDTA and no secretion or reabsorption in the circulation occurs.[86] Its plasma or serum concentration is less dependent on the muscular mass, inflammatory diseases, gender or diet, and these properties make it a good measure of the GFR compared to the traditional measurement of the SCr.[92, 93] As a result of this finding, several prediction equations have been derived from both pediatric and adult patients to estimate the GFR from the serum CysC concentration.[94, 95] Most of the studies that compared the CysC levels or CysC-derived equations with gold standard methods found CysC to be superior or at least equivalent to SCr.[93] Some studies on selected patient groups with either reduced or rapidly changing muscle mass also demonstrated that CysC is a sensitive marker of the GFR independent of body composition.[92]

Renal transplant recipients are a target group for whom the precise determination of GFR is crucial. Allograft function following renal transplantation is commonly monitored using SCr. However, plasma creatinine is far from being an ideal marker of the GFR, despite its convenience and low cost. Since the first publication in 1998,[96] quite a few original clinical papers have addressed the question of the use of CysC in kidney transplantation. A good number of studies identified serum CysC (or CysC-based equations) as a promising, easily measurable marker to estimate the GFR with a higher diagnostic value than SCr (or creatinine-based equations) and 24-hour creatinine clearance for evaluating the GFR in the follow up of adult kidney transplant patients.[97-99] Very recently, Masson *et al*[99] validated both CysC-based CKD-EPI equations (2012) in 670 kidney transplant recipients and concluded that both performed better than the serum creatinine-based CKD-EPI equation (2009).

A drawback of the use of CysC in kidney transplantation is the routine use corticosteroids. Glucocorticoid medication can compromise the use of serum CysC in this population and it is important to take this into account when interpreting this serum marker. Glucocorticoid therapy is one of the few identified circumstances that have an impact on the production of CysC in a dose-dependent manner, leading to systematic underestimation of the GFR.[100] Very large doses of glucocorticoids have been described to increase the production of CysC,[100, 101] whereas continuous low and medium doses of glucocorticoids

do not seem to alter the production of CysC.[102, 103] However, underestimations of GFR occur in some studies, e.g., with steroid dependent asthmatic patients.[104] Hence, moderate and high-dose glucocorticoids can limit the usefulness of CysC soon after kidney transplantation.[105]

For kidney transplant patients, early detection of decreased renal function is crucial so that measures to prevent further decreases in graft function can be taken. For this reason, the role of this marker in detecting post-transplant renal damage earlier than SCr has been investigated.[106-110] During the early post-transplantation period, the serum CysC decreases more rapidly than creatinine.[107, 111] As previously stated, glucocorticoids increase CysC concentrations and may lead to underestimation of the GFR; however, in stable renal graft recipients with low-dose immunosuppressive therapy, CysC is strongly correlated with the GFR and detects a GFR impairment earlier than SCr or creatinine-based eGFR.[100, 106, 107, 111-115]

In a prospective study of 30 consecutive patients with end-stage renal disease undergoing renal transplantation, Le Bricon and coworkers[107] evaluated CysC as a marker of allograft function during the early postoperative transplantation period. Serum CysC was more sensitive than SCr for detecting decreases in the GFR and predicting DGF. Furthermore, a more prominent rise in the plasma CysC values allowed for a more rapid diagnosis of acute rejection or treatment nephrotoxicity with the potential for more timely intervention. A prospective study performed by Thervet *et al* [106] in another 30 renal transplant patients also found that CysC allowed for earlier diagnosis of renal function recovery than SCr, particularly in patients with DGF. These findings were also confirmed by Hall and coworkers[108] in a cohort of 78 deceased-donor renal recipients, which showed that CysC outperformed SCr as a predictor of poor early graft function and the need for dialysis within the first week of kidney transplantation. Additionally, these authors demonstrated that CysC was a good prognostic marker of graft function at 3 months. In a recent article, Liu *et al* [110] evaluated the clinical value of CysC for the diagnosis of an acute rejection episode after renal transplantation in 76 recipients and concluded that CysC can predict an acute rejection episode after renal transplantation. In a recent multicenter study,[99] CKD-EPI formulae were compared for their accuracy in estimating the GFR, as determined by the gold standard, inulin clearance, in adult kidney transplant recipients (n=670) with stable graft function. This study used centralized, standardized assays for CysC and creatinine. Despite immunosuppressive treatment, formulae based on CysC and the combination of CysC and creatinine were less biased, more accurate and precise than the CKD-EPI-creatinine formula.

Because renal tubular impairment diminishes or precludes the ability to catabolize CysC, measuring CysC in urine can be a good estimator of renal tubular dysfunction. This was

observed in a prospective multicenter study of 91 deceased-donor kidneys transplants.[109] Serial urine samples were collected for 2 days following transplant and on the first postoperative day urine CysC was a predictor of DGF and of 3-month allograft function. In summary, CysC either in serum or urine displays several good characteristics that make it a practical and reliable biomarker for the early detection of DGF. Among the markers addressed in this review, serum CysC is likely the most commonly used biomarker as well as the closest to the clinical validation in kidney transplantation.

NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN

Neutrophil gelatinase-associated lipocalin is one the most promising and extensively studied biomarkers of acute kidney injury in a variety of acute clinical settings.[116-129] Neutrophil gelatinase-associated lipocalin, also known as human neutrophil lipocalin or lipocalin-2, is a glycoprotein that belongs to the lipocalin family. Originally, NGAL was identified in neutrophils covalently bound to gelatinase, but this lipocalin is also expressed at low levels in other human tissues including the kidney, lung, liver and epithelial cells in response to various pathologic states.[130] Human NGAL exists as a 25-kDa monomer, with a 45-kDa homodimer and is conjugated to gelatinase as a 135-kDa heterodimeric form, which is normally the main cellular source of circulating NGAL.[130]

As stated by one of the main researchers of these molecules, "*Lipocalins are small proteins that cells send out to bind things and carry them back*".[131, 132] Effectively, this family comprises several proteins, such as α1-microglobulin, retinol-binding protein 4, prostaglandin D synthase, and nitrophorines, which are specialized in binding and transporting small hydrophobic molecules, such as vitamin A, free heme and heme groups that are complexed with nitric oxide.[133-136]

The main features of NGAL were described by Goetz *et al* [131, 137], who discovered that the most important ligands of NGAL were siderophores, which are small iron-binding molecules. Bacteria produce siderophores to scavenge iron from the extracellular space and use specific transporters to recover the siderophore iron complex, ensuring their iron supply. These findings were consistent with the most important function attributed to this lipocalin, which is the inhibition of bacterial growth by the inhibition of iron-binding molecules that are important to specific bacteria.[131, 137] Thus, NGAL behaves as a bacteriostatic agent in acute infections and, under physiological conditions, bacterial infections represent the most common condition associated with marked increases in the NGAL levels.[138] But, beyond its microbial effect NGAL seems to have more complex activities.[128, 139] Some systemic diseases that are not necessarily associated with infection were also associated with increased levels of serum NGAL, confirming that many other

tissues may express and release NGAL as an acute-phase factor signaling a condition of sustained injury, which is the case for inflammatory processes involving skin, intestine and certain types of cancer, like adenomas and inflamed epithelia of the bowel, adenocarcinomas of the breast, and urothelial carcinomas.[140-149] Renal tubular injury is another pathologic state that induces the expression of NGAL and that increases its levels by approximately 1000-fold, which is rapidly apparent in both the urine and serum.[128, 139, 150] The relatively rapid time course of NGAL changes with respect to renal injury in comparison to SCr levels is one of the main advantages of NGAL, which makes this a superior or complementary biomarker in the diagnosis and prediction of acute kidney injury.[117, 151-158]

The genesis and sources of serum and urinary NGAL in response to renal injury is a subject under study. It was demonstrated that NGAL exists in two separate body pools: the systemic and the renal pools. In the steady state, NGAL is normally expressed at very low concentrations in multiple cell types. Accordingly, in healthy individuals, NGAL is detectable in the systemic circulation only at low levels. In the kidney, circulating NGAL is filtered in the glomerulus and luminal NGAL is completely reabsorbed in the proximal tubule by a megalin-dependent pathway. Hence, only traces of NGAL are detectable in urine. During injury or inflammatory processes, NGAL is massively released from activated neutrophils and the urinary levels correlate with serum levels independent of the cause of increased NGAL production. However, when massive NGAL quantities are excreted in the urine this usually indicates injury and damage to the proximal tubular cells due to ischemia-reperfusion injury, hypoxia, nephrotoxins or chronic progressive changes.[128, 150, 159] These kidney insults cause failure of absorption of the filtered NGAL leading to particularly high NGAL levels in urine, which is potentiated by the increased expression and secretion of NGAL from the nephron epithelia and from distant organs mainly the liver and the lungs.[126, 128, 150, 160-163]

Ischemia-reperfusion injury is an inevitable consequence of the kidney transplantation procedure and can be considered as a form of post-transplantation acute kidney injury. For this reason, several studies have investigated the utility of NGAL for the diagnosis and prognosis of acute graft dysfunction following kidney transplantation, with promising results.[69, 71, 72, 164-169] The values of NGAL collected shortly after renal transplantation were shown to predict the dialysis requirement within the first week, preceding the postoperative peak in SCr levels that typically does not occur before two to four days.[69, 71, 72, 164, 167, 170] Recently, the prognostic value of NGAL on graft function at one-year post-transplantation was also examined.[72, 171, 172] Different investigators reported consistent findings that NGAL may become one of the most important next-generation biomarkers in the diagnostic and clinical fields of acute graft dysfunction in renal transplantation (Table 1).

Table 1. NGAL in kidney transplantation: results from the main studies

Reference	Year	Material/ Methods	Study	Reports
Mishra [169]	2006	Recipients: 25 pediatric Donor: deceased and living Sample material: biopsies obtained from kidneys grafts within 1 h after reperfusion	Single center Prospective Single marker	Patients developing DGF showed the most intense NGAL staining during the previous biopsy
Parikh[164]	2006	Recipients: 53 adults and pediatric Donor: deceased and living Sample material: urine Number of sample collections: 1 Timing: within first 24 h post-kidney transplantation Definition of DGF: yes	Single center Prospective Multimarker (NGAL and IL18)	↑ NGAL values in DGF Urinary NGAL and IL-18 are predictive biomarkers of DGF (AUC=0.90 for NGAL)
Hall [71]	2010	Recipients: 91 adults Donor: deceased Sample material: urine Number of sample collections: 6 Timing: every 6h (first 2 days) Definition of DGF: yes	Single center Prospective Multimarker (NGAL,IL18,KIM1) No long-term follow-up (up to 3 months only)	↑ NGAL values in DGF Urinary NGAL and IL-18 are predictive biomarkers of DGF
Bataille [69]	2011	Recipients: 41 adult Donor: living and deceased Sample material: plasma Number of sample collections: 6 Timing: pre, post-KTx (first week) Definition of DGF: yes	Single center Prospective Single marker No long-term follow-up	↑ NGAL values in DGF Plasma NGAL level early and accurately predicted DGF after KTx
Hollmen [173]	2011	Recipients: 176 adults Donor: deceased Sample material: urine Number of sample collections: 6 Timing: pre and post-KT (days 1, 3, 7 and 14) Definition of DGF: yes	Single center Prospective Single marker Long-term follow-up (1-year)	Day 1 urinary NGAL predicted DGF but not long-term function
Kusaka [167]	2012	Recipients: 67 adults Donor: living and deceased Sample material: serum Number of sample collections: 5 Timing: pre-KTx and subsequent 4 days after KTx Definition of DGF: yes	Single center Retrospective Single marker	Serum NGAL on first day is highly sensitive and specific marker predicting future graft function
Hall [171] Extension	2012	Recipients: 153 adults Donor: deceased Sample material: urine Number of sample collections: 6 Timing: every 6 h (first 2 days) Definition of DGF: yes	Multicenter Prospective Multimarker (NGAL, IL-18) Long-term follow-up (1-year)	Perioperative urine NGAL and IL-18 are associated with poor 1-year graft function
Lee [170]	2012	Recipients: 59 adults Donor: Deceased and living Definition of DGF: yes	Single center Retrospective Multimarker (NGAL, IL-18)	NGAL is an early and sensitive marker of graft dysfunction while IL18 showed limited values

KTx: Kidney transplantation; DGF: delayed graft dunction; NGAL: Neutrophil gelatinase-associated lipocalin; IL-18: interleukin-18; AUC: Area under the curve.

OXIDATIVE STRESS

Oxidative stress is one of the most important components of the ischemia-reperfusion process.[174-176] It reflects an imbalance between reactive oxygen species (ROS) and cellular mechanisms for detoxifying the reactive intermediates or for repairing the resulting damage. Disturbances in the normal state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell.

In kidney transplantation, oxygen free radicals are the most likely agents responsible for initiating the damage associated with reperfusion injury.[177] Oxygen free radicals or, more generally, ROS are products of normal cellular metabolism. The average person has approximately 10,000–20,000 free radicals attacking each body cell every day. Free radicals are defined as molecules or molecular fragments containing one or more unpaired electron in atomic or molecular orbits, which gives a considerable reactivity to the free radical. The well-known radicals derived from oxygen, such as superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) represent the most important class of radical species generated in living systems.[178]

In physiological conditions, ROS are produced specifically to serve essential biological functions, such as defense against infections. In these conditions, the rates of free radical production and elimination are equal, leading to a steady state that is presumably tolerated by the cell. The antioxidant defense mechanisms can be divided into two major groups: i) endogenous, which are mainly enzymes, such as superoxide dismutases (SOD), catalases, glutathione reductases (GR) and peroxidases (GPx); ii) and small molecules, which are mostly exogenous and act as free radical scavengers (vitamins A, C, and E, carotenoids and polyphenol).[178] In some pathological conditions, an imbalance between ROS generation and the antioxidant capacity leads to enhanced ROS activity and oxidative stress. When these antioxidant mechanisms cannot counterbalance the levels of free radicals generated, cell damage and tissue injury can take place.[179]

Reactive oxygen species may cause tissue injury via several mechanisms. Because they are potent oxidizing and reducing agents, ROS directly damage cellular membranes and modify several biological molecules, such as lipids, proteins, and nucleic acids. The byproducts of these reactions can serve as biomarkers of oxidative stress.[174, 175] Of the many biological targets of oxidative stress, lipids are the most involved class of biomolecules. Lipid oxidation generates a huge variety of secondary products, including reactive carbonyl compounds like malondialdehyde (MDA). This aldehyde is the principal and most studied product of polyunsaturated fatty acid peroxidation, and, for this reason, MDA is a marker that is widely used to assess lipid peroxidation.[178, 179] Markers of

oxidative stress, including elevated levels of MDA and reduced antioxidant activity, have been reported in renal patients.[180, 181] There is substantial literature on oxidative stress and renal disease but data about kidney transplantation in the early stages are scarce. The restoration of kidney function after transplantation can lead to improvement of the oxidative stress, but some studies report increased systemic biomarkers of oxidative stress in kidney transplant recipients,[182, 183] specifically in the early phase of transplantation[184, 185] and, thereafter, coexisting with chronic allograft tubular atrophy/interstitial fibrosis.[182, 186]

It has long been suspected that oxidative stress contributes to the injury of ischemic and reperfused tissues.[187] During the ischemic phase of ischemia-reperfusion injury, there is a depletion of ATP followed by a rapid increase in ROS, including superoxide, during reperfusion.[188-190] In the setting of kidney transplantation, not only are there ischemic and reperfusion periods required for the preservation and implantation procedures but the placement of the kidney into an immune milieu can also act as an adjuvant for oxidative damage. Specifically in kidney transplants from deceased donors, brain death is associated with generalized ischemia due to a hyperactivity of the sympathetic system, which aims to maintain the cerebral perfusion pressure. During this ischemic phase, there is a depletion of the oxygen supply to renal cells and the endothelial and tubular epithelial cells are particularly vulnerable to oxygen depletion.[191-194] Warm ischemia after kidney vessels clamping and the cold ischemia after refrigeration also reduce the oxygen and nutrients supply to tissues. Reperfusion of the ischemic kidney further worsens the state of oxidation with additional release of free radicals. Hence, ROS are generated during both phases of ischemia-reperfusion.[187, 195]

As in other clinical conditions, if the scavenging capacity of the kidney is lower than the excessive ROS generated during ischemia-reperfusion injury, such oxidative imbalance may trigger a robust inflammatory response within the transplanted organ, leading to cellular destruction, tissue damage and graft dysfunction.[185, 196] Thus, severe reperfusion injury is a risk factor for DGF and the detection of ROS could be an early warning of graft injury. Waller and coworkers studied blood samples in porcine kidney allografts before and after reperfusion injury and demonstrated that both plasma carbonyl and 8-isoprostane (products of protein and lipid damage by free radicals, respectively) could be reliable biomarkers for predicting reperfusion injury.[197] To the best of our knowledge, no similar studies were conducted on this topic in human kidney transplantation.

A wide range of antioxidant enzymes may potentially exert a protective influence by limiting the production of ROS and the damage of oxidative stress following ischemia-reperfusion injury of a kidney graft. The kidney has naturally occurring antioxidant enzymes to counteract the effects of oxygen free radicals. Superoxide dismutase

catalyzes the conversion of superoxide to the harmless hydrogen peroxide. Glutathione works in a similar manner, but it can also act on organic peroxides.[198, 199] Oxygen free radical damage occurs despite the presence of scavengers indicating that the protective ability of these scavengers is overwhelmed.[200, 201] There is some evidence that during the early phase of ATP depletion that typically occurs in ischemia, manganese superoxide dismutase (MnSOD), a major mitochondrial antioxidant that eliminates superoxide, is inactivated.[202] Some studies performed on heart transplantation (animal model), have revealed a decrease in the antioxidant protein levels and a sequential loss in enzyme activity for MnSOD, catalase and GPx in acutely rejecting cardiac allografts.[203, 204] To the best of our knowledge, there are no similar studies in humans.

Conflicting results are reported in the literature on the activities of antioxidant enzymes in kidney transplant patients. Glutathione compounds and SOD have been reported to increase[205, 206] decrease[183] or not change[207] following renal transplantation. Whitin *et al* [205] reported a rapid increase in the plasma GPx activity after transplantation. The plasma GPx activity was two times higher 3 days after transplantation in adult patients who received a kidney transplant from a related donor and rapidly increased over the first 2 weeks post-transplant in adult recipients from a deceased-donor and pediatric patients undergoing kidney transplantation from related donors. Zachara *et al* [206] have shown that plasma GPx activity increases rapidly 3 days after renal transplantation and doubles two weeks later. Both of these studies suggested that monitoring plasma GPx might be a useful marker for monitoring the transplanted kidney function and a valuable tool for the postoperative detection of DGF.

Not only in the early post-transplant period but also in the longer-term, oxidants and antioxidants can be biomarkers of graft dysfunction with diagnostic accuracy. Oxidative stress is believed to be a common pathway that leads to both immunological and nonimmunological stress in the setting of kidney transplantation and to the development or progression of chronic allograft nephropathy. Increased plasma and intragraft levels of MDA and reduced antioxidant activity were found in kidney allografts with chronic tubular atrophy/interstitial fibrosis, which suggests the possibility of early detection even when graft dysfunction is undetectable with serum creatinine [182, 186, 208]. Our understanding of oxidative stress has significantly advanced in the last decade, but these experimentally derived ideas have yet to be fully integrated into clinical practice.

LEPTIN AND ADIPONECTIN

White adipose tissue is now recognized as a multifunctional organ.[209] In addition to its central role in lipid storage, white adipose tissue has a major endocrine function by

synthesizing a multitude of protein cytokines termed adipokines. Leptin and adiponectin are two adipokines that elicit generally opposing effects. Epidemiologic studies have highlighted the associations between hyperleptinemia and several metabolic and inflammatory factors involved in the development of cardiovascular disease, whereas adiponectin may possess antiatherogenic properties in individuals with normal kidney function, exhibiting a reverse association with cardiovascular risk.[209-212] In chronic kidney disease the clinical significance and prognostic implications of leptin and adiponectin are not well understood. Uremic patients have increased circulating levels of both adipokines, which may result from an increase in their production and/or decrease in their renal clearance.[213-222]

Leptin is one of the best-known adipokines. It is a small 16-kDa peptide that is mainly but not exclusively produced in the adipose tissue. It is a cytokine that is known to participate in multiple cellular and physiological processes and is currently believed to be involved in the regulation of appetite and energy expenditure.[223] Under physiological conditions, leptinemia directly reflects the amount of body fat,[224] and although not thoroughly demonstrated, renal clearance is considered to be the major route for leptin metabolism.[214, 219] Some studies performed in animal and human models strengthen this hypothesis.[214, 225-227] Cumin and coworkers[226] have realized that bilateral nephrectomy in rats decreased the plasma leptin clearance by approximately 81%. Immediately after removing both kidneys, leptinemia increased approximately 4-fold after one hour and almost 10 times after four hours. In comparison, SCr also increased after the removal of both kidneys and doubled after 2 hours. These findings were validated in humans in a study by Zeng *et al*[227] that also demonstrated that leptin is promptly and efficiently cleared from plasma by renal extraction.

Adiponectin is a 28-kDa protein that is produced almost exclusively by adipocytes. In contrast to other adipokines, adiponectin is a paradoxical adipokine because circulating levels of it are decreased in subjects with obesity, diabetes mellitus and atherosclerosis. An inverse correlation between adiponectin and kidney function has been reported and, as in the case of leptin, the plasma levels of adiponectin are elevated in kidney failure,[213, 218, 228, 229] suggesting that the kidneys play a role in the biodegradation and/or elimination of this adipokine.[216]

The role of the kidney in the biodegradation of leptin and adiponectin appears to be confirmed in studies conducted in kidney transplantation, where the circulating levels of these adipokines decreased after successful transplantation.[230-235] Kokot *et al* [231] found normalization of leptinemia in the early post-transplant period. Landt *et al* [235] reported that the plasma leptin levels decreased to less than half the pre-transplant values 6 days after successful renal transplantation, reaching the levels obtained from the normal healthy

adults. Another study from Howard *et al* [236] also found lower values of leptinemia in kidney transplantation recipients than in dialysis patients, although the values remained higher than in controls. Based on this growing evidence that leptin and adiponectin are primarily removed from circulation by the kidney, we hypothesized, to our knowledge for the first time, that higher levels of leptin and/or adiponectin could indicate poorer clearance and could be an early biomarkers of graft dysfunction, expressed as DGF.

Chapter 3

Material and Methods

Content

MATERIAL AND METHODS

Materials and methods are described in the original publications (I to VI), but due to the word limit of the manuscripts some additional and more detailed information about the procedures and methodology are presented here.

3.1. STUDY DESIGNS

This study involved three cohorts of kidney transplantation patients comprising two retrospective cohorts and one prospective cohort.

Studies I and VI
Retrospective cohort studies

Studies II, III, IV, and V
Prospective cohort studies within the first 7-days after kidney transplantation and one-year follow-up data on adverse outcomes.

3.1.1. RETROSPECTIVE COHORT STUDIES (STUDIES I AND VI)

Study samples

Two retrospective cohorts of kidney transplant patients were studied; all adult recipients with more than one-year of follow-up after kidney transplantation, who were identified retrospectively from an electronic database from August 4, 1983 to December 31, 2008 (first retrospective cohort used for study I, n=1273) and from August 4, 1983 to December 31, 2012 (second retrospective cohort used for study VI, n=1281) were eligible for study inclusion.

Exclusion criteria
Recipients younger than 18 years old or who required multiorgan transplantation were excluded. Study VI (the second retrospective cohort) excluded also retransplants and recipients from living donors.

Data collection

Since July 1983, the Department of Nephrology and Kidney Transplantation of Centro Hospitalar do Porto routinely registers the demographic and clinical data from each transplant in this center or received from other centers, providing a large cohort of renal transplant recipients available for follow-up studies. This database includes the following data: donor variables (gender and age at the time of death or kidney donation); recipient variables (age and gender, primary cause of renal disease, modality and duration of pre-transplant dialysis, previous transplant history (first or prior), and panel-reactive antibodies); transplantation-related factors (living or deceased donor, number of HLA A, B, and DR mismatches between the donor and recipient, and the cold ischemia time); and some post-transplantation features (induction and maintenance immunosuppressive regimen, history of DGF, occurrence of acute rejection, status of kidney allograft, cause of graft loss, vital status, and cause of death).

This database did not include information related to the post-transplant period, namely graft function within the first year. Therefore, to develop SCr as a surrogate marker for long-term graft survival study (study I), SCr levels at 1, 6 and 12 months were collected purposely by the author, from the paper or electronic medical records of each patient and registered in the electronic database. Additionally, for the purpose of this research, during 2009 (study I) and 2014 (study VI) the recipients registered in the database were checked and updated according to death, readmission to dialysis or retransplantations. The patient and graft follow-up times were then calculated.

3.1.2. PROSPECTIVE COHORT STUDIES (STUDIES II TO V)

Study sample

The prospective cohort included 40 recipients who received transplants from December 2010 to May 2011 (studies II to V).

Exclusion criteria

Recipients younger than 18 years old or who required multiorgan transplantation were excluded from enrollment. After transplantation, recipients with primary graft failure were also excluded.

Sample size

The prospective study was deemed exploratory. When the study protocol was designed, some of the candidate biomarkers were little studied or previously unstudied as early markers of kidney graft dysfunction. Given this, it would be imprudent and costly to recruit a larger cohort, particularly because each patient was subjected to five measurements of each marker. Therefore, based on costs, clinical and statistical issues the suitable sample size was set to 40 patients, representing half a year of the kidney-only transplant activity of our center.

Recruitment procedures

The recruitment of patients included in the prospective cohort lasted 6 months, from December 2010 to May 2011. During this time, consecutive patients with end-stage renal disease, who were undergoing living or deceased donor kidney transplantation in the Department of Nephrology and Kidney Transplantation of Centro Hospitalar do Porto were prospectively enrolled. On the day of transplantation (day 0), participants who met the inclusion criteria were asked to participate in the study by the nephrologist who called and received the patient for transplant surgery. This nephrologist, who was also part of research team, was responsible for explaining the objectives of the study as well as its risks and benefits. A briefing written explanation about the study was then given to each patient, and those who decided to take part signed the consent form, which was also dated and signed by the nephrologist researcher. After the information that a transplant was ongoing and the patient had agreed to participate, the mechanics of the study was initiated.

Biological sample collection, preparation and storage

Five total blood and urinary samples were collected per patient for the determination of biomarkers as follows: 3 to 6 h prior to transplant surgery (pre-transplant); on the morning following surgery, approximately 8 to 12 h after graft reperfusion (day-1); and then on the second, fourth, and seventh days after transplant.

Blood and urine samples were collected using conventional procedures, using ethylenediaminetetraacetic acid (EDTA) as anticoagulant or not, and were centrifuged shortly afterward. All of the serum, plasma, and urine samples were aliquoted and frozen within one hour after collection and were stored at -80°C until analysis. Erythrocytes were obtained by centrifugation of whole blood for 5 minutes at 5000 rpm and then plasma was removed. Then, erythrocytes were washed three times with 0.9% NaCl solution and centrifuged at 2000 rpm for 5 minutes after each wash. The whole blood samples,

erythrocytes and separated plasma/serum were aliquoted within one hour after collection and stored at -80ºC until assayed. All determinations were performed approximately one to two weeks after collection.

Biomarker analyses/measurements

Creatinine was measured as routine in serum (SCr) using a kinetic colorimetric assay based on a compensated Jaffé method using a calibrator for automatic systems (Cobas 6000, Roche Diagnostics, Germany).

Cystatin C was measured in serum via a particle-enhanced nephelometric immunoassay (Siemens Diagnostics, Germany). The normal range for this assay is 0.53-0.95 mg/l.

Neutrophil gelatinase-associated lipocalin was measured in urine (uNGAL) using a two-step chemiluminescent microparticle immunoassay on a standardized clinical platform (ARCHITECT, Abbott Diagnostics, Germany) and the normal upper limit for uNGAL in healthy volunteers is 132 ng/ml (95th percentile) with this assay. The coefficient of variation for this automated assay has been reported to be ≤5% and sensitivity (uNGAL concentration corresponding to a coefficient of variation of 20%) was found to be <2 ng/ml.

Adipokines

Leptin was measured in serum using ELISA with kits from Mercodia (Uppsala, Sweden). Mercodia Leptin ELISA is based on the direct sandwich technique in which two highly specific monoclonal antibodies are directed against separate antigenic determinants on the leptin molecule. As for all assays, each laboratory should collect data and establish its own range of expected values and the reference ranges considered in our study were 2 to 5.6 ng/mL for males and 3.7 to 11.1 ng/mL for females.

Adiponectin was measured in plasma using Mercodia kits (Uppsala, Sweden). Mercodia Adiponectin ELISA is a solid phase, two-site enzyme immunoassay. It is based on the sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the adiponectin molecule. The normal concentrations were typically 3 to 300 ng/mL.

Oxidative Stress Markers

Malondialdehyde was measured in fresh plasma samples using a commercial high-performance liquid chromatography kit (Chromsystems, Munich, Germany). Preparation of samples was performed according to the manufacturer's instructions. A derivatisation step in which protein bound to MDA is hydrolyzed and converted into a fluorescent probe (60 min at 95°C) was performed for the determination of MDA. The fluorescent probe was then cooled (2-8°C), centrifuged, mixed with a reaction solution and injected into the high-performance liquid chromatography system. The isocratic separation via HPLC at 30°C using a “reversed phase“ column takes 4 minutes for each sample. The chromatograms were recorded with a fluorescence detector. The quantification was performed with the delivered calibrator, and the concentration was calculated via integration of the peak heights. The MDA values are given in µmol/L for plasma and the reference range is 0.77-0.93 µmol/L.

Total Erythrocyte Superoxide Dismutase activity was measured in erythrocytes according to the protocol of Beauchamp and Fridovich using the RANSOD kit (Randox Laboratories, UK).[237] This method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. The activity value of SOD is expressed in U/g hemoglobin and the reference range is 1102 - 1601 U/g hemoglobin.

Glutathione reductase was measured in plasma/serum using a kit supplied by Randox Laboratories Ltd. (Cat. No. GR2368) according the manufacturer's recommendations. Glutathione reductase is required for the regeneration of reduced glutathione, which is important for normal cellular metabolism. This enzyme is often discussed in association with glutathione peroxidase, which requires reduced glutathione for activation. Glutathione reductase catalyzes the reduction of glutathione in the presence of NADPH, which is then oxidized to $NADP^+$. The oxidation of NADPH to NADP+ is accompanied by a decrease in the absorbance at 340 nm, providing a spectrophotometric means of detection that is directly proportional to the GR activity in the sample. The reference range is 33 to 73 U/L plasma/serum.

Glutathione Peroxidase was measured in whole blood using a kit supplied by Randox Laboratories Ltd. (Cat. No. RS505) and the appropriate whole blood control (SC692). Whole blood (50 µl) was diluted with 1 ml RANSEL diluting agent and incubated for 5 min. One ml of double-strength Drabkin’s solution was added, and assays were performed within 20 min. The GPx activity was measured at 340 nm, using a sample volume of 5 µl

in a total reaction volume of 285 µl based on the Paglia and Valentine method. The Randox Ransel reference range for GPx activity is 27.5 - 73.6 U/g Hb.

The *Total Antioxidant Status* (TAS) was measured in serum by total antioxidant quantification using ABTS+ (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) radical formation with the commercially available Randox Total Antioxidant Status test kit (Ref. NX2332, Randox Lab., Ltd., UK). The ABTS was incubated with peroxidase (metmyoglobin) and H_2O_2 to produce the blue-green colored radical cation ABTS+, which is measured at 600 nm. The antioxidants in the added sample cause the suppression of this color to a degree that is proportional to their concentration. The reference range was based on a European working population going from 1.30 to 1.77 mmol/L in plasma.

3.2. CLINICAL DEFINITIONS

The *pre-transplant time on dialysis* was calculated since the beginning of dialysis until the date of transplantation; in retransplants the time on dialysis between transplants was added.

Expanded-criteria donors refer to older kidney donors (≥60 years) or donors 50 to 59 years old with at least two of the following three features: history of hypertension, terminal SCr higher than 1.5 mg/dl, or death from a cerebrovascular event.

The level of *"HLA mismatches*" was used to indicate the number of human leukocyte antigens at the A, B, and DR *loci* found in the donor that were not present in the recipient.

Acute rejection was defined as either biopsy-proven rejection or anti-rejection treatment without biopsy.

Delayed graft function was defined according to United Network for Organ Sharing as the requirement for dialysis within the first seven days after renal transplantation due to the absence or irrelevant improvement in graft function. Complementarily, graft function was considered "*prompt or immediate*" (non-DGF) if no dialysis was required during the first week after transplantation.

3.3. ETHICS

Both the retrospective and prospective studies were approved by the Institutional Review Board of Centro Hospitalar do Porto, which conducted a scientific and ethical evaluation, and were performed in accordance with the national rules and regulations as well as international guidelines. All patients included in the prospective studies gave informed consent to participate and data were de-identified by removing the patient names and hospital numbers.

3.4. STATISTICAL ANALYSIS

The statistical analyses performed in each study are described in each article. Some of the statistical methodologies were purposely learned and trained for this work, and then an overview of these methodologies and the reasons for their use are explained in more detail here.

Linear mixed effect model

The studies II, III and IV had a longitudinal design and used the *Linear Mixed Effect Model* as statistical methodology. A longitudinal study generally yields multiple or “repeated" measurements on each subject. The main interest of this type of study is typically to characterize the way the outcome or potential exposures changes over time, and the predictors of the change. Multiple observations from the same subject are likely to be positively correlated and cannot be regarded as independent from each other. The observations at level 1 (biomarker measurements) are clustered at level 2 (patients), generally making them correlated (fig. 2). Thus, specific statistical methods should be used to analyze longitudinal data.

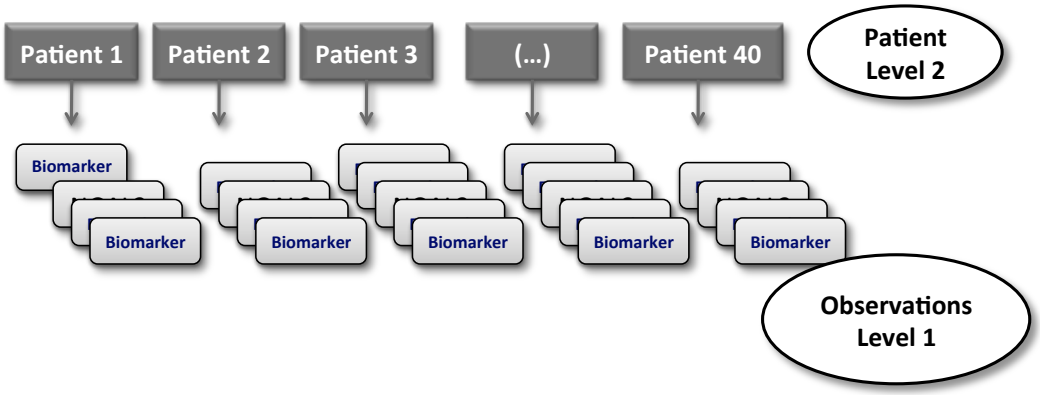


Fig. 2: Example of an unbalanced repeated-measures study

Traditionally, most researchers consider a repeated analysis of variance (ANOVA) when they plan to analyze multiple observations from each subject. Generalized linear models (GLM), such as ANOVA and analysis of covariance (ANCOVA), are the most commonly used procedures to examine changes across time. However, these methods would accurately estimate the model parameters in only a balanced repeated-measures design (e.g., equal group sizes). This condition is not easy to fulfill in a clinical study and the use of traditional univariate and multivariate test statistics may increase Type I errors in the

condition of an unbalanced repeated-measures design.[238, 239] Furthermore, in ANOVA the error variance, which characterizes the within-subjects variance, and the variance of the random effects, which characterizes the between-subjects variance, are treated as being homogeneous across subject groups or levels of covariates. When this homogeneity of variance assumption is violated, as it can happen with unbalanced data, the results of the analysis may not be trustworthy.[240]

We used linear mixed-effects models with a maximum likelihood estimation to evaluate the changes in the nine studied biomarkers over time. These models, also known as individual growth curve models,[238, 241] random regression models,[242, 243] hierarchical linear models,[244, 245] or multilevel models[246] are extremely flexible and offer several advantages over traditional methods for the analysis of longitudinal data. First, this statistical technique does not require balanced data, which provides a more adaptable and powerful approach when handling unbalanced data (e.g., unequal sample size, inconsistent time interval, and missing data).[238, 247] In our study, the number and spacing of measurement occasions were fixed (i.e., the same points in time for all individuals); however, missing measurements were identified within individuals, primarily regarding in uNGAL, because of the lack of urine samples in recipients with transient anuria. Missing data are a critical problem in clinical research. The use of a linear mixed model overcomes the limitation of other conventional statistical techniques that do not allow for missing observations. Both standard univariate GLM and multivariate analysis of variance (MANOVA) GLM require complete data. Thus, even if a single time-point measure is missing, the subject is discarded. With a few missing observations a great deal of data can be lost. One of the main advantages of mixed models is that we can use all existing data. If a score is missing, it is just missing. It has no effect on the other scores from the same patient.

A second advantage of these models is that they enable the study of both intra and interindividual differences in the growth parameters (e.g., slopes and intercepts). These individual differences can be modeled by assuming different intercepts and slopes for each subject that is by adding an additional random effect for the subject. These random effects reflect each person's growth or development across time, and the variance of these random effects indicate the degree of variation that exists in the subjects.[240] In the mixed effects modeling approach, factors may be considered to have both fixed and random effects, whereas in the GLM, each factor must be considered either a fixed or random effect. Thus, in linear mixed models each recipient is assigned a different intercept value that is estimated by this type of modeling. This approach enables the non-independence of measures to be determined by assuming a different "baseline" value for each subject.[248] This approach is valuable, for example, in the field of uNGAL and serum

CysC because individuals vary not only in their initial status but also their rates and pattern of changes. Most methods for repeated-measure designs (e.g., multiple regression analyses, ANOVA, MANOVA) only focus on group differences in the patterns of change; however, variations in growth curve parameters may also exist at the individual level. When we have a design in which we have both fixed-effect parameters associated with one or more continuous or categorical covariates and random effects that are associated with one or more random factors, we have what is often referred to as a mixed model. The fixed-effect parameters describe the relationships of the covariates to the dependent variable for an entire population, and the random effects are specific to the clusters or subjects within a population.[238, 247, 249]

Third, the estimation of the parameters of a statistical model is a key step in most statistical analyses and linear mixed model analyses estimate the parameters with greater precision, primarily when the number of time points increases. As previously discussed the mixed model equations are solved to obtain the fixed and random effects and error terms, which produce shrinkage estimators.[250] This approach improves the reliability of the growth parameters by reducing the standard errors of the within-subject change in the growth parameter estimates.[238, 247, 251]

Fourth, the effects of predictors on individual changes can flexibly be added in the growth curve models, which can be used to explore the causal links between the association of predictors and changes in outcome variables across time.[244] This approach enables the inclusion of discrete or continuous predictors in the model, as well as time variants or time invariants. Time-variant predictors refer to independent variables that change over time (e.g., body mass index), whereas time-invariant predictors refer to independent variables that remain constant over time (e.g., gender or pre-transplant time on dialysis).

Finally, mixed models for longitudinal data can model differences in variances, and not just means, across subjects and time-varying covariates. Thus, linear mixed models are more powerful than other methods (e.g., ANOVA, MANOVA, multiple regression analyses) in examining the effects associated with repeated measures because they can model the covariance matrix (i.e., fitting the covariance structure to the data) rather than imposing a certain type of structure as commonly used in traditional univariate and multivariate approaches. Sphericity or compound symmetry in the model can be assumed; however, we can also allow the model to select its own set of covariances or use different, and often more logical covariance structures.[252-255]

Survival analysis considering competing risks

Survival analysis is commonly used in medical research and typically focuses on time to event data.[256] Events are typically designated as "*failures*", and the time to the event is designated as the "*survival time*". This "*survival time*" is the time that takes an individual to fail because of a particular event, which is measured from an origin of time; it can be characterized by the hazard function, which represents the rate of occurrence of the event at a given time *t*, but primarily via the survival function, which represents the probability of *surviving* up to time *t*, that is, the probability that the event has not yet occurred before time *t*. If the patient does not have an event before the end of the study, the patient's event time is right censored. In the presence of non-informative right censoring and given a random sample of observed individuals, both functions are empirically estimable through consistent quantities such as the Nelson-Aalen estimator for the hazard function or the Kaplan-Meier estimator (1-KM) for the survival function.[256, 257] In kidney transplantation the 1-KM is one of the most used methods to study graft and patient survival, which censors all but one type of outcome (typically graft failure or patient death). Thus, the probability of an event of interest (e.g. graft failure) is estimated in an ideal world where the other types of events do not exist (e. g. patient death). However, when competing risks are present these methods produce biased estimates of endpoint probabilities because they do not account for the various types of outcomes that can occur.[258, 259]

In several clinical research settings the need to address multiple potential outcomes is nearly ubiquitous. Thus, several causes of failure are possible but the occurrence of one event precludes the occurrence of the other events (e.g. when failures are different causes of death, only the first cause can be observed).[260] This situation is known as competing risks. When a competing risks situation is present, each subject can experience one of a number of different events and we are interested in the time to the event, as well as the event type.[258]

In kidney transplantation, for example, graft loss can be defined as the absence of kidney function, which occurs any time after transplantation because of patient death or irreversible graft injury that requires chronic dialysis and/or retransplantation. After a kidney transplant, all recipients are at risk of both mutually exclusive events. Thus, competing risks are said to be present. Only the first event that occurs is observed, which is typically referred to as the "cause of failure", and the occurrence of one event will prevent the other event from ever occurring. If a patient returns to dialysis because of the loss of a functioning graft, the other failure may, in fact, be observable (death after graft failure); however, this event is no longer the event of interest for the kidney transplantation setting, because the patient is now a "dialysis patient" (fig. 3). Traditionally, only one event

is chosen for analysis (graft failure or patient death), and the competing risk event is ignored and treated as a non-informative right-censored observation; furthermore, classical survival methods, such as Kaplan-Meier, are used for inference. However, this approach leads to bias and the true rate of the event is overestimated.[258, 261]

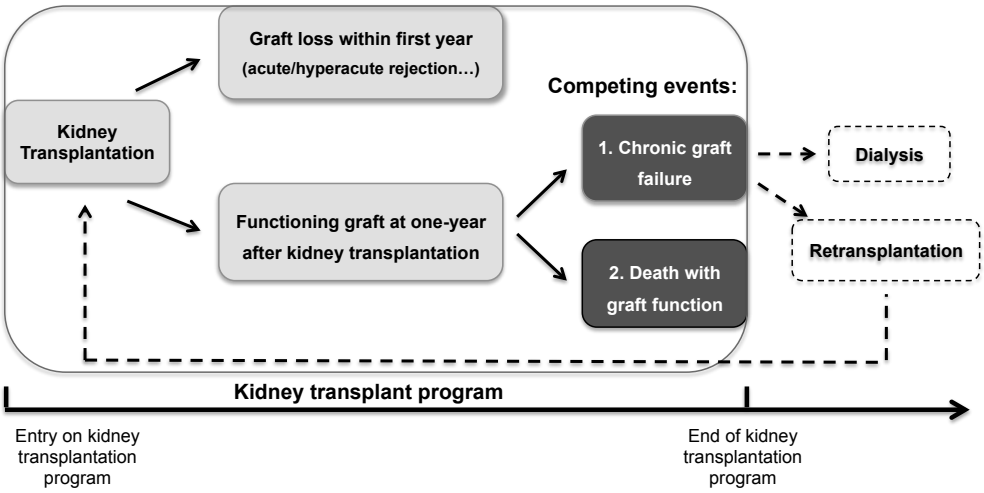


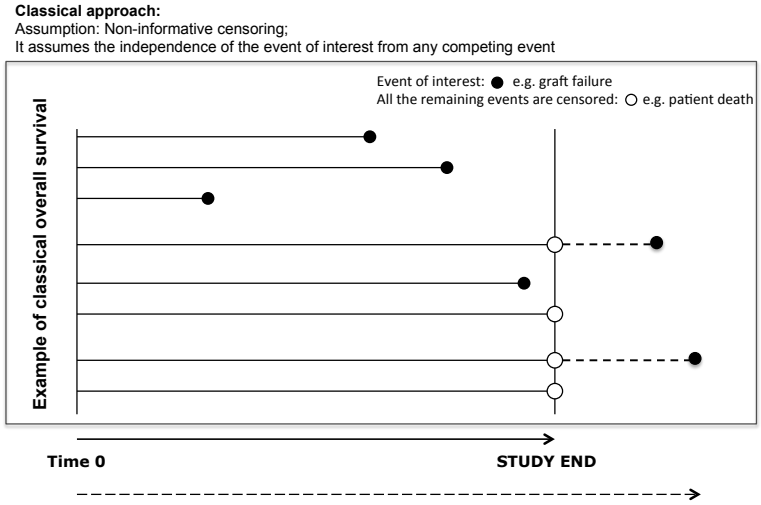
Fig. 3: The distinctive outcomes after kidney transplantation

Cumulative Incidence

The estimation of the cumulative incidence function is of primary interest in most clinical studies. The Kaplan-Meier (KM) method can be used to obtain a nonparametric estimate of the cumulative incidence when the data consist of subjects who experience an event and the censoring mechanism is assumed to be non-informative (independent of the event time, or in other words, when the time at which a subject experiences an event is assumed to be independent of a mechanism that would cause the patient to be censored).[262] Thus, unless the competing risk event is independent of the main event, the classical KM method and other standard methods would provide biased estimators for the different probabilities of interest. Furthermore, as discussed by Tsiatis,[263] the independence between distinct causes of failure cannot be checked on the basis of the competing risks observed data.

Specific methods are thus required for the estimation of survival probabilities in the presence of competing events. Several authors have discussed the topic of competing risks and the estimation of the cumulative incidence of an event. Gooley *et al*[264] discussed the appropriateness of the cumulative incidence function (CIF) in competing risks analysis as opposed to routinely using the 1-KM estimator. The CIF is defined as the probability of an individual experiencing an event by time *t* when other competing risks are acting on the individual. The authors pointed out that 1-KM is a function of the hazard for failures due to

the cause of interest only and do not depend on the hazard for failures due to the competing risks. Therefore, 1-KM is not interpretable as an estimate of the probability of failure as a result of the cause of interest when competing risks are present. In practice, the 1-KM treats patients who experience the competing risk prior to the event of interest as censored. Therefore, they are 'redistributed to the right' which incorrectly assumes that the cause of interest is still possible beyond the time at which the censoring occurred (fig. 4).[261]



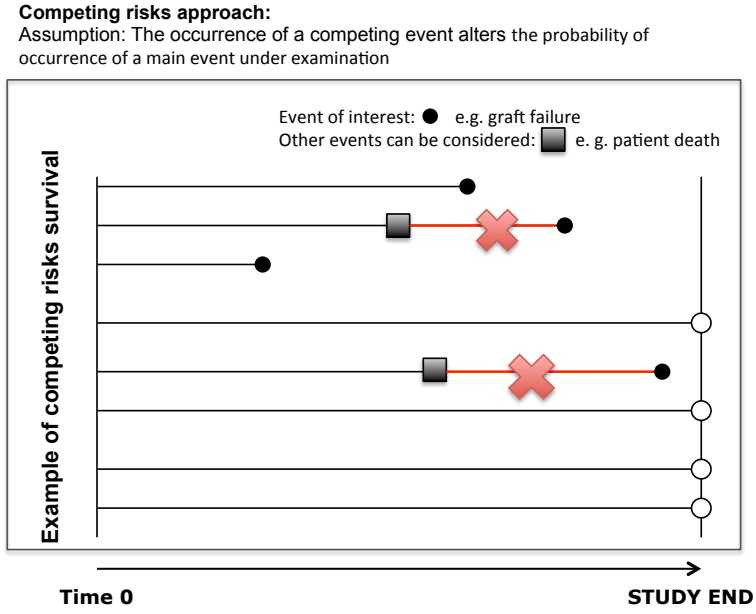


Fig. 4: Survival analysis: classical vs. competing risks approaches.

In kidney transplantation, if the study event is, for example, graft failure as a result of the loss of functioning, the KM method attempts to predict what the graft failure probability would be if no patient ever died. This value would be higher than the actual value; however, to do this, KM method relies on the assumption of noninformative censoring for not only the patients who are alive, but also for the patients who have died, which is an assumption that is not true in this situation or in many other medical applications. Thus, the typical 1-KM estimate, which includes patient death that occurred prior to graft failure as non-informative censoring, attempts to estimate the probability of graft failure if no individual died. Therefore, the typical KM method applied to a competing risks situation overestimates the probabilities of graft failure and is thus inappropriate in the presence of competing risks (Fig. 4).[258, 261, 262, 265, 266]

Regression analysis for competing risks

There are two popular methods for regression analysis when competing risks are present: regression on the cause-specific hazards using the competing risks analogue to the Cox proportional hazards model and the regression model for the cumulative incidence function proposed by Fine and Gray.[265]

The most commonly used method is a Cox model stratified on different competing risk events. This model is based on the cause-specific hazard function, which measures the instantaneous failure rate as a result of one risk at a time. It is routinely estimated by constructing the Cox models on cause-specific hazards and treating time to event from the other competing risks as censored. For each risk, the effects of prognostic factors are assessed as constant hazard ratios on the instantaneous failure rate of this risk.[267]
An important function in the competing risk framework is the CIF. However, many situations require more than a summary measure; some form of regression would be useful. The models based on CIF resemble a more “real world” situation and it is directly related to crude survival rates; thus, it is essential to decision makers. To estimate failure in the presence of competing risks, Fine and Gray[265] proposed a proportional hazards model for the CIF, and this regression model has been widely used in medical research.[268-271]

In the current study, the influence of DGF was evaluated using the cause-specific Cox proportional hazards regression model (based on the cause-specific hazard) and the Fine and Gray model (based on the subdistribution hazard) to analyze how the effects of this

covariate differed between these two approaches. Both results are valid; however, their interpretations are different and depend on the purpose of the study (etiology vs. prediction) as discussed in the manuscript of the study VI.[266, 270, 272]

Chapter 4

Results

Content

4.1. STUDY I

SERUM CREATININE AS A SURROGATE ENDPOINT FOR LONG-TERM GRAFT SURVIVAL

Fonseca I, Almeida M, Martins LS, Santos J, Dias L, Lobato L, Henriques AC, Mendonça D. First-year renal function predicts long-term renal allograft loss. *Transplant Proc*. 2011; 43(1):106-12.

When the true endpoints occur late the use of good surrogate markers can substantially reduce the study duration because they are obtained sooner at a lower cost or less invasively than the long-term clinical efficacy endpoint. When using a surrogate endpoint, one would like to make the same inference as if a true endpoint has been observed.

Among the renal transplant community a natural candidate for long-term graft loss is renal function, and some studies noted that graft function at one-year is a strong surrogate marker of late graft outcome. Because international trends might not be reflected in local populations and each center must assess what works best in their local reality, this study was performed to examine the predictive effect of renal function within the first year (expressed as the SCr level at 1, 6 and 12 months as well as the changes between those months) on the long-term graft survival, and to identify other independent factors that are associated with late failure in renal transplants. The main goal of this study was to establish SCr within the first year after kidney transplantation as a surrogate endpoint for the subsequent prospective studies that intended to identify markers of kidney graft dysfunction in the immediate postoperative period as follows:

Novel Biomarkers	>>	Surrogate Endpoint	>>	Definitive Endpoint
uNGAL CysC Leptin/Adiponectin Oxidative stress		SCr at 6 months SCr at 12 months		Graft failure Patient Death

Studies II to V **Study I**

The main results are expressed below:

- Among a large number of variables that were taken into consideration, the SCr levels at 1, 6 and 12 months following kidney transplantation as well as changes between 1 and 6 months and between 6 and 12 months were independently associated with late graft failure using Cox multivariable analysis.

- Other significantly factors associated with late graft failure were a younger age of the recipient, older age of the donor, acute rejection, female gender, adjusting for other factors usually associated with graft loss, such as pre-transplant time on dialysis, HLA mismatches, and DGF occurrence.

First-Year Renal Function Predicts Long-Term Renal Allograft Loss

I. Fonseca, M. Almeida, L.S. Martins, J. Santos, L. Dias, L. Lobato, A.C. Henriques, and D. Mendonça

ABSTRACT

Purpose. We performed a retrospective study to examine the impact on long-term graft survival of first-year posttransplantation renal function, as evaluated by serum creatinine.

Patients and Methods. We analyzed data from 1,273 adult kidney transplants performed between 1983 and 2008. All recipients >18 years old were included if their grafts had survived beyond 1 year, excluding patients simultaneously transplanted with other organs. Cox proportional hazards multivariable analysis was used to examine the relationship between first-year posttransplantation renal function and death-censored graft loss, adjusted for other variables. Renal function in the first year was expressed as serum creatinine levels at 1, 6, and 12 months as well as the change in creatinine between those 3 periods.

Results. Posttransplantation 1-month serum creatinine levels and change between 1 and 6 months were independent predictors of long-term graft loss. Multivariable analysis also identified donor age (increasing), acute rejection episode occurrence, recipient age at transplantation (decreasing), and gender (female) as independently predictive of graft failure, adjusting for other factors usually associated with graft loss, namely, pretransplantation time on dialysis, HLA mismatches, and delayed graft function. The predictive effect of creatininemia was sustained at 6 and 12 months, after adjusting for these covariates.

Conclusions. Posttransplantation serum creatinine levels at 1, 6, and 12 months were independent predictors of graft survival, suggesting that they could be considered as surrogate endpoints for long-term death-censored graft loss.

Renal transplantation is the treatment of choice for patients with end-stage renal disease. In the past two decades, progress in surgical procedures, medical care, and immunosuppression have significantly improved the short- and long-term results of organ transplantation.[1,2] Nevertheless, grafts continue to fail over time, and chronic dysfunction remains the leading cause of late allograft loss among surviving recipients.[3]

One of our concerns is to obtain insight into the factors associated with long-term allograft survival to identify early markers of chronic allograft dysfunction as well as potential interventional pathways. Long-term graft survival is an ideal endpoint, but evaluating an outcome in the long term usually requires a time-consuming process. For this reason, an easier approach seeks to identify alternative endpoints or short-term markers that predict long-term survival and therefore can be considered as potentially useful surrogates. This approach is widely used in clinical research on cancer and cardiovascular disease, and recently it has been applied in the context of renal disease.[4]

There has been a research focus among the transplant community to obtain a diagnostic marker that may serve as a surrogate for eventual graft loss.[5–7] A natural candidate for this surrogate endpoint is renal function. Creatinine levels and changes in creatinine levels within the first year after transplantation have been shown to be important

From the Renal Transplant Unit of Nephrology Department, Centro Hospitalar do Porto, Hospital de Santo António (I.F., M.A., L.S.M., J.S., L.D., L.L., A.C.H.), Unidade Multidisciplinar de Investigação Biomédica (I.F., M.A., L.S.M., J.S., L.L., A.C.H.); and Instituto de Ciências Biomédicas Abel Salazar and Instituto de Saúde Pública, Universidade do Porto (D.M.), Porto, Portugal.

Address reprint requests to Isabel Fonseca, Serviço de Nefrologia Centro Hospitalar do Porto, Hospital de Santo António, Largo Prof. Abel Salazar, 4099-001 Porto, Portugal. E-mail: isabelf27@gmail.com

0041-1345/11/$–see front matter
doi:10.1016/j.transproceed.2010.12.034

parameters influencing and predicting long-term graft survival.[7–12] However, not all studies support the validity of serum creatinine as a predictor of long-term graft loss. Serum creatinine underestimates deterioration of glomerular filtration rate and changes only occur at a late stage in the progression of graft dysfunction. For these reasons, some authors consider creatinine to be a poor predictor of both chronic allograft dysfunction and graft loss.[13–15]

The present retrospective analysis of a single-center experience was intended to examine the predictive effect on long-term graft survival, of renal function within the first year, as expressed by serum creatinine level at 1, 6, and 12 months and the change between those months, as well as to identify other independent factors associated with late graft failure. This study is the first attempt of our Unit to develop a surrogate endpoint for subsequent studies that will intend to identify earlier sensitive markers of renal allograft dysfunction.

SUBJECTS AND METHODS

Study Population and Data Collection

This study of 1,273 kidney transplants performed at the Renal Transplantation Unit of Centro Hospitalar do Porto, Hospital Santo António, Porto between August 4, 1983, and December 31, 2008 excluded recipients of multiorgan transplants (kidney/pancreas and kidney/liver), pediatric patients (<18 years old at transplantation), and losses within the first year. Recipient, donor, and transplantation characteristics were mainly obtained from our electronic database, which holds longitudinal information of all renal transplantations since 1983. The following data were collected: donor variables (gender and age at time of death or kidney donation); recipient variables (gender, primary cause of renal disease, pretransplantation time on dialysis, age at time of transplantation, first or prior transplantation, and panel-reactive antibodies); transplantation-related factors (living or deceased donor, number of human leukocyte antigen (HLA) A, B, and DR mismatches, and cold ischemia time); and posttransplantation features (immunosuppressive regimen, history of delayed graft function, occurrence of acute rejection, status of kidney allograft, cause of graft loss, vital status, and cause of death). Serum creatinine levels at 1, 6, and 12 months were obtained from the medical records and from the hospital electronic laboratory database. All patients were followed from transplantation until death, readmission to dialysis, retransplantation, or May 2010. The study was approved by our Institutional Review Board. Data were deidentified by removing patient names and hospital numbers.

Definition and Categorization of Variables

Pretransplantation time on dialysis was calculated from the beginning of dialysis to the date of transplantation; in retransplants we added the time on dialysis between transplants.

The term "mismatch" was used for the number of HLA antigens that donor and recipient did not have in common. Female donor mismatch was labeled when a male recipient received a kidney from a female donor, so patients were grouped as female donor to male recipient or all other combinations (female to female, male to male, or male to female). Donor O blood group mismatch was considered when a non-O blood type person was a recipient of a kidney from an O donor blood type, so patients were grouped as O donor to non-O recipient or all other combinations.

Delayed graft function was defined by the need for dialysis in the first week after transplantation owing to an absence or a significant improvement in graft function. Acute rejection episode was defined as either biopsy-proven rejection or antirejection treatment without biopsy. Graft survival was censored for patient death with a functioning allograft or the end of the study (May 2010). Graft loss was defined as return to dialysis or retransplantation without returning to dialysis. Late graft loss was defined as graft loss beyond the first-year follow-up.

The sample was divided into 4 cohorts based on times in which immunosuppressive medications were introduced into clinical practice: era 1, before 1990, azathioprine and cyclosporine (no microemulsion); era 2, between 1990 and 1995, cyclosporine microemulsion; era 3, between 1996 and 2000, mycophenolate mofetil introduction and wide use of antithymocyte globulin; and era 4, after 2000, sirolimus availability and wide use of tacrolimus.

Renal function in the first year after-transplantation was expressed as serum creatinine level at 1, 6, and 12 months, as well as the change in creatinine from 1 to 6 months (ΔCr_{1-6}) and from 6 to 12 months (ΔCr_{6-12}). Serum creatinine levels were also categorized as: <1, 1–1.5, 1.5–2.0, 2.0–2.5, and ≥2.5 mg/dL.

Statistical Analyses

Continuous variables are expressed as mean values (standard deviation) and categoric variables as proportions. Graft loss due to chronic allograft failure was the endpoint. For this reason, graft survival was censored for patient death with a functioning allograft. Statistical analysis of death-censored graft survival was performed using several multivariable Cox proportional hazard models. Time to event (graft loss) incorporated in models excluded the first year after transplantation, owing to time dependency of serum creatinine during that year. Multivariable models were built both in a stepwise hierarchic manner, testing the significance of added terms by using the forward likelihood ratio method, and by using the entry method in a second block to guarantee that donor, recipient, and transplantation variables recognized to influence allograft survival in the current literature would be included and the effects adjusted for all variables in the model.

Recipient age (continuous and grouped), gender, pretransplantation time on dialysis, number of transplants performed (>1 vs 1), donor age (continuous and grouped); source of donor organ (living vs cadaveric donor), number of HLA mismatches, female donor mismatch (female donor–male recipient vs all other combinations), O donor blood type mismatch (O blood type donor vs all others), transplantation era, occurrence of delayed graft function, and occurrence of an acute rejection episode were the variables adjusted in the multivariable graft survival analysis. Recipient age was treated as continuous (years of age) and as a categoric variable (age higher or less than 55 years and age quartiles). The quartiles of recipient age were tested because of the small number of recipients >55 years at the time of transplantation.

Serum creatinine levels were included in the models as both continuous and categoric variables, the relative difference in serum creatinine level from 1 to 6 months (ΔCr_{1-6}) and from 6 to 12 months (ΔCr_{6-12}) as continuous variables. To avoid colinearity, serum creatinine levels at 1, 6, and 12 months were used in different models, as well as change of levels between 1 and 6 and between 6 and 12 months. We tested potentially interacting variables. Prespecified multiplicative interactions included serum creatinine levels by history of delayed graft function and occurrence of an acute rejection episode. Cox analyses are expressed as hazard rates (HR)

and 95% confidence intervals (CIs). All statistical analyses were performed by using the Statistical Package for the Social Sciences, version 18.0 (SPSS, Chicago, IL, USA). A P value of $<.05$ was assumed to indicate a statistically significant association.

RESULTS

Patient Characteristics

The demographic and clinical characteristics of the 1,273 adult renal transplants patients who met the criteria for inclusion and were enrolled are summarized in Table 1. At the time of transplantation, the mean recipient age was 40.7 years (range, 8–75 y). Almost 11% of patients were retransplants; only 6.1% received a kidney from a living donor. The mean number of HLA mismatches was 3.1 ± 1.6. There were 171 patients (13.4%) who underwent transplantation before 1990 (time of azathioprine and cyclosporine, no microemulsion), 339 (26.6%) between 1990 and 1995 (time of cyclosporine microemulsion), 320 (25.1%) between 1996 and 2000 (time of mycophenolate mofetil and antithymocyte globulin), and 443 (34.8%) after 2000 (time of tacrolimus and sirolimus).

The causes of graft loss are shown in Table 2. Chronic allograft dysfunction occurred in 249/416 graft failures (59.9%); it was the leading cause of kidney allograft loss. Recipient death the second cause of late failure, occurred in 149 patients (35.8%), and 857 patients (67.3%) still had functioning allografts at the censoring date.

Factors Affecting Long-Term Survival of the Grafts after the First Year

Proportional hazards Cox regression was used to investigate independent factors affecting long-term survival of the grafts that had reached 1 year to estimate the HR of graft failure. To assess the independent association of renal function in the first year after transplantation and long-term graft loss, several multivariable models were built. Owing to colinearity, serum creatinine levels at 1, 6, and 12 months were tested in various models, as well as ΔCr_{1-6} and ΔCr_{6-12}.

Table 1. Patient Demographic and Clinical Characteristics (n = 1,273)

Characteristic	
Donor	
Donor age (y, mean ± SD)	34 ± 15.7
Donor age ≥55 y (n, %)	162 (12.8)
Living donor (n, %)	78 (6.1)
Recipient	
Recipient age (y, mean ± SD)	40.7 ± 11.9
Recipient age ≥55 y (n, %)	185 (14.5)
Recipient age 1st quartile (<32 y, n, %)	320 (25.1)
Recipient age 2nd quartile (32–40 y, n, %)	319 (25.1)
Recipient age 3rd quartile (40.1–50 y, n, %)	316 (24.8)
Recipient age 4th quartile (>50 y, n, %)	318 (25.0)
Recipient female (n, %)	505 (39.7)
Previous dialysis time (y, mean ± SD)	4.2 ± 3.9
Preemptive (n, %)	18 (1.4)
Retransplants (n, %)	135 (10.6)
HLA mismatches (mean ± SD)	3.1 ± 1.6
After transplantation	
Delayed graft function history (n, %)	384 (30.2)
Acute rejection occurrence (n, %)	293 (23.0)

Table 2. Causes of Graft Loss

Characteristic	n (%)
Graft lost	416 (32.7)
Death with functioning graft	149 (11.7)
Graft functioning at May 31, 2010	857 (67.3)
Reason for graft lost (n = 416)	
Recipient death	149 (35.8)
Chronic allograft dysfunction	249 (59.9)
Recurrent disease	8 (1.9)
Rejection after suspending immunosuppression	4 (1.0)
Other	6 (1.5)

Model Development

All variables known to potentially influence long-term graft survival were considered for inclusion in the multivariable models. Accordingly, recipient age, gender, pretransplantation time on dialysis, and number of transplants performed (>1 vs 1), donor age, source of donor organ (living vs cadaveric donor), number of HLA mismatches between donor and receptor, female and O donor blood type mismatches, transplantation era, occurrence of delayed graft function and occurrence of an acute rejection episode were the covariates entered into the multivariable Cox proportional hazards models in addition to 1-month serum creatinine levels and ΔCr_{1-6}.

On the basis of these models, we observed that the 1-month serum creatinine levels and ΔCr_{1-6} were significantly associated with death-censored graft failure (HR for each 1 mg/dL increase at 1 month, 1.909; 95% CI, 1.622–2.246; and HR for each 1 mg/dL increase between 1 and 6 months, 1.832; 95% CI, 1.535–2.186; Table 3). The analyses also identified increasing donor age, female recipient, and history of acute rejection episode as significant predictors of graft failure, as well as decreasing age of the recipient. Donor age, treated as a continuous variable, showed a borderline effect on graft loss (HR for each 1 year increase, 1.020; 95% CI, 1.010–1.029; $P < .001$), but considering a cutoff of 55 years, in a separate model, donors >55 years old were 2.5-fold more likely to be associated with graft failure due to chronic dysfunction compared with donor <55 years old (HR, 2.5; 95% CI, 1.7–3.6; $P < .001$). In relation to recipient age, which was included as a continuous variable, the hazard of long-term graft loss decreased with age (HR, 0.975; 95% CI, 0.962–0.989; $P < .001$). This observation was also sustained when the recipient age was divided into quartiles: The first and second quartiles were significantly associated with chronic graft loss (<32 y and 32–40 y, respectively). Using the cutoff value of recipient age lower versus higher than 55 years, as widely used in the literature, the effect of recipient age was not significant.

Table 3. Multivariate Cox Proportional Hazard Analysis of the Hazard of Long-Term Allograft Loss Censored for Death (Including Serum Creatinine Level at 1 month and ΔCr_{1-6})

	Long-Term Allograft Loss Censored for Death		
	HR*	95% CI	*P* Value
Serum creatinine level at 1 mo (1 mg/dL increase)	1.909	1.622–2.246	<.001
ΔCr_{1-6} (1 mg/dL increase)	1.832	1.535–2.186	<.001
Donor age (1 y increase)	1.020	1.010–1.029	<.001
Acute rejection (occurrence vs no occurrence)	1.591	1.189–2.128	.002
Recipient age (quartiles; reference, 4th quartile)			.007
1st vs 4th quartile (<32 vs >50 y)	2.249	1.397–3.621	.001
2nd vs 4th quartile (32–40 vs >50 y)	1.911	1.183–3.086	.008
3rd vs 4th quartile (40.1–50 vs >50 y)	1.541	0.921–2.578	.099
Recipient gender (female vs male [reference])	1.373	1.031–1.827	.030

*Adjusted for pretransplantation time on dialysis, transplantation era, number of transplants performed (first vs regraft), source of donor organ (living vs cadaveric donor), number of HLA mismatches, history of delayed graft function (yes vs no), female donor mismatch (female donor to male recipient vs all other combinations), and donor O mismatch (O blood type donor to non-O receptor).

Other factors, including pretransplantation time on dialysis, number of transplants, female donor, O donor blood type mismatch, number of HLA mismatches, transplantation era, and delayed graft function, were not significantly associated with graft failure, after adjusting for the other variables in the model. We excluded from the model potentially interacting variables owing to nonsignificant effects: namely, the occurrence of delayed graft function or an acute rejection episode with serum creatinine levels.

The predictive effect of serum creatinine was maintained when levels at the first month were replaced by those at 6 and 12 months (Tables 4 and 5). Adjusting for the same variables sixth-month serum creatinine level and ΔCr_{6-12} were significantly associated with death-censored graft failure: HR for each 1 mg/dL increase at 6 months, 2.407; 95% CI, 2.019–2.870; and HR for each 1 mg/dL increase between 6 and 12 months, 2.080; 95% CI, 1.736–2.492 (Table 4). Similar findings were verified with 1-year serum creatinine levels, which were also significantly associated with death-censored graft failure: HR for each 1 mg/dL increase at 1 year, 2.267; 95% CI, 1.935–2.589; (Table 5).

An additional analysis used the variables previously incorporated with serum creatinine as a categoric variable, ie, serum creatinine levels <1 mg/dL considered as the reference grouping class. Considering serum creatinine at 1 month, only levels ≥2.5 mg/dL were significantly associated with graft loss (HR, 2.502; 95% CI, 1.096–5.712; $P < .03$), but at 6 and 12 months, serum creatinine ≥1.5 mg/dL had predictive effects for graft failure (Table 6).

DISCUSSION

Over the past 30 years, improvements in the prevention and treatment of acute rejection episodes and infections have raised first-year kidney transplantation survival to >90%. Nevertheless, a steady decline in graft survival beyond the first year still occurs; many kidneys are lost owing to chronic dysfunction.

Research has focused on finding sensitive and predictive markers to identify patients at high risk for chronic graft dysfunction, seeking to adjust procedures and hopefully extend kidney life. Owing to the need for long follow-up times, surrogate endpoints for late allograft failure are lacking. Clinically, chronic graft dysfunction describes the inexorable decline in renal function with time. Serum creatinine levels within the first year after transplantation

Table 4. Multivariate Cox Proportional Hazard Analysis of the Hazard of Long-Term Allograft Loss Censored for Death (Including Serum Creatinine Level at 6 months and ΔCr_{6-12})

	Long-Term Allograft Loss Censored for Death		
	HR*	95% CI	*P* Value
Serum creatinine level at 6 mo (1 mg/dL increase)	2.407	2.019–2.870	<.001
ΔCr_{6-12} (1 mg/dL increase)	2.080	1.736–2.492	<.001
Donor age (1 y increase)	1.020	1.010–1.029	<.001
Acute rejection (occurrence vs no occurrence)	1.484	1.110–1.985	.008
Recipient age (quartiles; reference, 4th quartile)			.005
1st vs 4th quartile (<32 vs >50 y)	2.302	1.423–3.725	.001
2nd vs 4th quartile (32–40 vs >50 y)	2.126	1.309–3.451	.002
3rd vs 4th quartile (40.1–50 vs >50 y)	1.677	0.991–2.838	.054
Recipient gender (female vs. male [reference])	1.341	1.006–1.787	.045

*Adjusted for pretransplantation time on dialysis, transplantation era, number of transplants performed (first vs regraft), source of donor organ (living vs cadaveric donor), number of HLA mismatches, history of delayed graft function (yes vs no), female donor mismatch (female donor to male recipient vs all other combinations), and donor O mismatch (O blood type donor to non-O receptor).

Table 5. Multivariate Cox Proportional Hazard Analysis of the Hazard of Long-Term Allograft Loss Censored for Death (Including Serum Creatinine Level at 1 Year)

	Long-Term Allograft Loss Censored for Death		
	HR*	95% CI	*P* Value
Serum creatinine level at 12 mo (1 mg/dL increase)	2.267	1.985–2.589	<.001
Donor age (1 y increase)	1.021	1.012–1.030	<.001
Acute rejection (occurrence vs no occurrence)	1.504	1.128–2.007	.005
Recipient age (quartiles; reference, 4th quartile)			.009
1st vs 4th quartile (<32 vs >50 y)	2.210	1.367–3.574	.001
2nd vs 4th quartile (32–40 vs >50 y)	2.112	1.303–3.423	.002
3rd vs 4th quartile (40.1–50 vs >50 y)	1.706	1.012–2.875	.045
Recipient gender (female vs male)	1.346	1.008–1.798	.068

Recipient gender was excluded from the model (P = .068).
*Adjusted for pretransplantation time on dialysis, transplantation era, number of transplants performed (first vs regraft), source of donor organ (living vs cadaveric donor), number of HLA mismatches, history of delayed graft function (yes vs no), female donor mismatch (female donor to male recipient vs all other combinations), and donor O mismatch (O blood type donor to non-O receptor).

have been used as an early marker and surrogate endpoint for long-term graft loss.[7–12]

We sought to analyze the ability of serum creatinine measurements at 1, 6, and 12 months to predict death-censored graft survival, to then substitute this distant clinical event seeking to identify early markers of graft dysfunction. Our analysis confirmed serum creatinine within the first year after transplantation to be an important independent parameter influencing long-term survival. We obtained similar results when treating serum creatinine levels as a categoric variable, observing an increased HR for graft loss for higher creatinine levels.

There are a number of ways to measure or estimate renal function. All have advantages and disadvantages regarding costs, feasibility, and accuracy. Serum creatinine is a simple, inexpensive, widely available, and easily reproducible parameter for both single-center and large multi-institutional studies. In addition, and as required for an ideal laboratory biomarker, an elevation of serum creatinine indicates kidney damage and precedes the onset of chronic graft dysfunction.[4]

Several retrospective studies have shown that elevated posttransplantation serum creatinine levels are associated with an increased risk of late graft failure.[7,11–13,16] Posttransplantation renal function within a few days after transplantation or at the time of discharge from the hospital has been associated with long-term survival.[11,12] Hariharan et al analyzed data from a large cohort of adult recipients who received renal transplants from living and cadaveric donors in the United States between January 1988 and December 1998.[7] Data were obtained from all 256 kidney transplant programs as reported to the Organ Procurement and Transplantation Network/United Network for Organ Sharing. They revealed a correlation between serum creatinine levels at 6 and 12 months and long-term graft failure. An increment of 1.0 mg/dL in serum creatinine at 1 year after transplantation increased the odds of graft failure by 63%. When this was accompanied by a change in creatinine of 0.5 or 1.0 mg/dL from 6 months to 1 year, the HR of graft failure increased to 2.26 and 3.13, respectively. Hariharan et al concluded that increases in serum creatinine at 1 year and enhanced changes between 6 and 12 months progres-

Table 6. Multivariate Cox Proportional Hazard Models of the Hazard of Long-Term Allograft Loss Censored for Death (Serum Creatinine Level Treated as Categoric Variable)

	Long-Term Allograft Loss Censored for Death		
	HR*	95% CI	*P* Value
Serum creatinine level at 1 mo (reference, <1 mg/dL)			
≥2.5 vs <1 mg/dL	2.502	1.096–5.712	.029
Serum creatinine level at 6 mo (reference, <1 mg/dL)			
1.5–2.0 vs <1 mg/dL	2.263	1.088–4.707	.029
2.0–2.5 vs <1 mg/dL	3.882	1.747–8.628	.001
≥2.5 vs <1 mg/dL	9.554	4.185–21.812	<.001
Serum creatinine level at 12 mo (reference, <1 mg/dL)			
1.5–2.0 vs <1 mg/dL	2.950	1.245–6.990	.014
2.0–2.5 vs <1 mg/dL	4.765	1.936–11.727	.001
≥2.5 vs <1 mg/dL	13.971	5.507–35.444	<.001

As in the models in Tables 3–5, donor age, recipient age and gender, occurrence of acute rejection, ΔCr_{1-6}, and ΔCr_{6-12} were significant predictors for graft survival (data not shown).
*Adjusted for pretransplantation time on dialysis, transplantation era, number of transplants performed (first vs regraft), source of donor organ (living vs cadaveric donor), number of HLA mismatches, history of delayed graft function (yes vs no), female donor mismatch (female donor to male recipient vs all other combinations), and donor O mismatch (O blood type donor to non-O receptor).

sively increased the risks of graft failure.[7] Kasiske et al also confirmed serum creatinine to be a predictor of graft failure in a study that evaluated the first decline in inverse creatinine as a surrogate marker for graft survival.[16] Serum creatinine values were collected over a maximum follow-up of 22 years among 101 consecutive renal transplant recipients; 30% chronic decline in inverse serum creatinine was reported to be a good predictor of late renal allograft failure.[16]

Serum creatinine has also been validated as a surrogate endpoint in kidney transplantation clinical trials.[17] Fitzsimmons et al demonstrated the predictive value of serum creatinine levels during the first 6–12 months, reporting rates of graft loss over 3 years of 19.3% and 17.0% for patients with serum creatinine values >1.5 mg/dL at 6 and 12 months, respectively.[17] For patients with serum creatinine levels >2 mg/dL at 6 and 12 months, the 3-year graft losses were 24.6 and 26.5%, respectively.[17] Paraskevas et al established that serum creatinine at 1 year after transplantation was the best predictor of graft survival, also corroborating this parameter as a surrogate endpoint for long-term outcomes after kidney transplantation.[18]

But not all studies agree with the validity of serum creatinine as a predictor of long-term survival. Some workers consider creatinine to be a flawed marker, owing to its variability by gender, race, recipient age, and body weight.[13,14] After adjusting for gender and age in our analysis, both donor and recipient serum creatinine levels had predictive effects on long-term posttransplantation survival. We did not adjust for race because almost all of our kidney recipients were white.

Several authors have reported that isolated values of serum creatinine lose significance in the Cox model when variables expressing evolution of the renal function are introduced.[8,19] Kaplan et al noted that the rate of change in graft function within the first year after tranplantation explained the poor predictive value of 1-year serum creatinine.[15] This proposal was not supported by our study. Isolated levels of serum creatinine at 1, 6, and 12 months maintained their significance even after including the course of creatinine in the first 12 months after transplantation, expressed as ΔCr_{1-6} and ΔCr_{6-12}.

Some clinical studies have reported that, creatininemia is a poor predictor of both chronic transplant dysfunction and long-term graft failure, because changes in serum creatinine occur at a late stage in the progression of graft dysfunction.[13,14] We agree that when serum creatinine starts to rise, chronic structural lesions are already present and it may be too late for effective intervention strategies. But as a predictor for long-term graft failure, this parameter can be used as a surrogate endpoint for prospective studies to identify early biomarkers to predict renal function decline, allowing earlier identification or prediction of chronic transplant dysfunction and/or occurrence of graft failure.

In addition to first-year renal function, we identified donor age, recipient age and gender, and history of acute rejection episodes as the most important factors predicting long-term kidney allograft survival. As expected, female recipients, recipients of grafts from older donors, and patients who experience acute rejection episodes were more likely to develop chronic graft dysfunction and graft failure than those without these conditions. Somewhat unexpectedly, the hazard of death-censored graft loss decreased with increasing recipient age at transplantation. This finding is not new in kidney transplantation.[20,21] We can not forget that, for all analyses, patient death with a functioning graft was not considered to be a graft failure, because the aim of the present study was identification of predictors for graft loss due to organ-related problems. In view of the fact that the most frequent cause of graft failure among older patients is death with a functioning allograft, the HR of graft loss decreased with recipient age. One-fourth of our patients were <32 years old at transplantation, so they had more time to develop chronic graft dysfunction than older patients. In contrast, recipients >50 years old had less time.

As widely known, delayed graft function can have a negative impact on both short- and long-term graft survival. In the present study, when the data were entered into a multivariable analysis, neither delayed graft function nor HLA mismatches predicted graft loss. It is possible that first-year graft function diluted the impact of delayed graft function, because serum creatinine levels were the strongest predictor of chronic graft dysfunction in all models.

Serum creatinine within the first posttransplantation year shares many features of surrogate endpoints accepted in other domains, including biologic plausibility and a strong relationship to the clinical endpoint. Our study confirmed that the association of serum creatinine with prognosis was independent of several other predictive factors. We think that these findings are important to establish creatinine levels as a surrogate endpoint that will reflect long-term renal transplant outcomes and be a reliable substitute for prospective studies designed to identify earlier and more sensitive markers of graft dysfunction.

In conclusion, in the search for new markers of graft dysfunction, it is of great significance to choose a suitable endpoint for analysis. The present study was the first approach to validate serum creatinine levels within the first year after transplantation as an alternative endpoint of late chronic graft dysfunction. The findings confirmed that renal function in the first year after transplantation can be regarded as a variable predicting long-term renal graft survival.

REFERENCES

1. Hariharan S: Long-term kidney transplant survival. Am J Kidney Dis 38(suppl 6):S44, 2001

2. Meier-Kriesche HU, Ojo AO, Port FK, et al: Survival improvement among patients with end-stage renal disease: trends over time for transplant recipients and wait-listed patients. J Am Soc Nephrol 12:1293, 2001

3. Kasiske BL, Gaston RS, Gourishankar S, et al: Long-term deterioration of kidney allograft function. Am J Transplant 5:1405, 2005

4. Lemley KV: An introduction to biomarkers: applications to chronic kidney disease. Pediatr Nephrol 22:1849, 2007

5. Hariharan S, McBride MA, Cohen EP: Evolution of end-points for renal transplant outcome. Am J Transplant 3:933, 2003

6. Hariharan S, Kasiske BL, Matas A: Surrogate markers for long-term renal allograft survival. Am J Transplant 4:1179, 2004

7. Hariharan S, McBride MA, Cherikh WS, et al: Post-transplant renal function in the first year predicts long-term kidney transplant survival. Kidney Int 62:311, 2002

8. Kasiske BL, Andany MA, Danielson B: A 30% chronic decline in inverse serum creatinine is an excellent predictor of late renal allograft failure. Am J Kidney Dis 39:762, 2002

9. Meier-Kriesche HU, Baliga R, Kaplan B: Decreased renal function is a strong risk factor for cardiovascular death after renal transplantation. Transplantation 75:1291, 2003

10. Pascual J, Marcén R, Zamora J, et al: Very early creatinine as a surrogate marker for graft survival beyond 10 years. J Nephrol 22:90, 2009

11. Govani MV, Kwon O, Batiuk TD, et al: Creatinine reduction ratio and 24 hour creatinine excretion on post transplant day two. Simple and objective tools to define graft function. J Am Soc Nephrol 13:1645, 2002

12. Cecka MJ, Terasaki PI, eds. The UNOS Scientific Renal Transplant Registry, in Clinical Transplants. Los Angeles: UCLA Tissue Typing Laboratory; 1998, P 1.

13. Chapman JR, O'Connell PJ, Nankivell BJ. Chronic renal allograft dysfunction. J Am Soc Nephrol 16:3015, 2005

14. Dominguez J, Kompatzki A, Noramuena R, et al: Benefits of early biopsy on the outcomes of kidney transplantation. Transplant Proc 37:3361, 2005

15. Kaplan B, Schold J, Meier-Kriesche H-U: Poor predictive value of serum creatinine for renal allograft loss. Am J Transplant 3:1560, 2003

16. Kasiske BL, Andany MA, Hernandez D, et al: Comparing methods for monitoring serum creatinine to predict late renal allograft survival. Am J Kid Dis 38:1065, 2001

17. Fitzsimmons W, Thompson D, Hariharan S, et al: Serum creatinine as a surrogate endpoint for graft loss in kidney transplantation validation efforts from multicenter trials. Am J Transplant 2(suppl 3):272 (abstract 533), 2002

18. Paraskevas S, Kandaswamy R, Humar A, et al: Predicting long-term kidney graft survival: can new trials be performed. Transplantation 75:1256, 2003

19. Gómez Marqués G, Errasti Goenaga P, Lavilla Royo FJ, et al: Evolution of the renal function is a better predictor of long-term survival than serum creatinine. Am J Transplant 37:3701, 2005

20. Boom H, Mallat MJK, de Fijter JW, et al: Delayed graft function influences renal function, but not survival. Kidney Int 58:859, 2000

21. Helal I, Abderrahim E, Ben Hamida F, et al: The first year renal function as a predictor of long-term graft survival after kidney transplantation. Transplant Proc 41:648, 2009

4.2. STUDY II

NGAL: A PROMISING MARKER OF GRAFT DYSFUNCTION AND A PREDICTOR OF ONE-YEAR GRAFT FUNCTION

Fonseca I, Oliveira JC, Almeida M, Cruz M, Malho A, Martins LS, Dias L, Pedroso S, Santos J, Lobato L, Henriques AC, Mendonça D. Neutrophil Gelatinase-Associated Lipocalin in kidney transplantation is an early marker of graft dysfunction and is associated with one-year renal function. *J Transplant*. 2013; 2013: 650123. doi: 10.1155/2013/650123.

When this study was designed only two studies on this topic had been published in the field of kidney transplantation.[164, 169] When the sample collection was initiated (December 2010) a third study was published[71] (Hall *et al.* 2010). In other words, very little was known at that time about the usefulness of NGAL in the renal transplant setting.

The primary goals were to examine the longitudinal changes of the uNGAL within the first week after kidney transplantation, evaluate uNGAL as an early marker of delayed graft function, and determine its association with one-year graft function. Urinary NGAL was the first biomarker studied within the prospective component of the PhD studies using the same cohort. Forty adult, consecutive patients with ERSD undergoing kidney transplant surgery were prospectively enrolled.

- Eighteen of them (45%) experienced DGF and displayed median uNGAL concentrations that were persistently higher at all measured timepoints compared than those in patients with immediate recovery of their graft function (non-DGF).

- The curve of reduction of the uNGAL levels appeared very different between patients with and without DGF. Shortly after transplantation, patients with DGF displayed a significant increase in the uNGAL levels reaching median levels higher than 800 ng/ml and a reduction from the fourth day. In recipients with prompt graft function, the uNGAL levels decreased immediately and rapidly until reaching median values below 150 ng/ml within the first 24 h after transplantation.

- The association of DGF with longitudinal changes in uNGAL was studied using a linear mixed-effects model, by controlling for variables that were found to be associated with uNGAL with bivariate analysis. The pre-transplant time on dialysis, time measurement of uNGAL and DGF were independently associated with the uNGAL levels, and the prompt function recipients had, on average, lower levels of uNGAL at all timepoints. According to our estimation, for a patient with a dialysis time of approximately 4.1 years, the initial

values of uNGAL (3-6 h after transplantation) are approximately 242 ng/mL higher in patients who went on to develop DGF, and these values increase even more in the subsequent days. A significant interaction between the time of measurement and DGF confirmed that longitudinal changes in the uNGAL levels depend on whether the recipient had or had not undergone DGF.

- Receiver-operating characteristic (ROC) curves showed uNGAL on the first postoperative days were moderately accurate in predicting DGF with areas under the ROC curves (AUC-ROC) of 0.77 and 0.88, respectively, 3 to 6 h and 8 to 12 h after surgery and were highly accurate for the second, fourth, and seventh day (0.96, 0.99 and 0.93, respectively). The diagnostic performance of uNGAL was better than of SCr at all timepoints and was quite similar to that of CysC.

- The prognostic value of early uNGAL values on long-term allograft function (one-year after kidney transplantation) evaluated via SCr was tested using multivariable linear regression analysis. Urinary uNGAL measured on the fourth and seventh days were independent predictors of one-year graft function, adjusting for established variables that usually affect graft function, including acute rejection episodes and re-hospitalizations that occurred during the first post-transplant year.

Hindawi Publishing Corporation
Journal of Transplantation
Volume 2013, Article ID 650123, 12 pages
http://dx.doi.org/10.1155/2013/650123

Research Article

Neutrophil Gelatinase-Associated Lipocalin in Kidney Transplantation Is an Early Marker of Graft Dysfunction and Is Associated with One-Year Renal Function

Isabel Fonseca,[1,2] José Carlos Oliveira,[3] Manuela Almeida,[1,2] Madalena Cruz,[3] Anabela Malho,[1] La Salete Martins,[1,2] Leonídio Dias,[1] Sofia Pedroso,[1] Josefina Santos,[1,2] Luísa Lobato,[1,2] António Castro Henriques,[1,2] and Denisa Mendonça[4,5]

[1] *Department of Nephrology and Kidney Transplantation, Centro Hospitalar do Porto, Hospital de Santo António, 4099-001 Porto, Portugal*
[2] *Unit for Multidisciplinary Investigation in Biomedicine (UMIB), 4099-003 Porto, Portugal*
[3] *Department of Chemical Chemistry, Centro Hospitalar do Porto, Hospital de Santo António, 4099-001 Porto, Portugal*
[4] *Institute of Public Health (ISPUP), University of Porto, 4050-600 Porto, Portugal*
[5] *Department of Population Studies, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, 4050-313 Porto, Portugal*

Correspondence should be addressed to Isabel Fonseca; isabelf27@gmail.com

Received 30 July 2013; Revised 7 September 2013; Accepted 20 September 2013

Academic Editor: Bruce Kaplan

Urinary neutrophil gelatinase-associated lipocalin (uNGAL) has been suggested as potential early marker of delayed graft function (DGF) following kidney transplantation (KTx). We conducted a prospective study in 40 consecutive KTx recipients to evaluate serial changes of uNGAL within the first week after KTx and assess its performance in predicting DGF (dialysis requirement during initial posttransplant week) and graft function throughout first year. Urine samples were collected on post-KTx days 0, 1, 2, 4, and 7. Linear mixed and multivariable regression models, receiver-operating characteristic (ROC), and areas under ROC curves were used. At all-time points, mean uNGAL levels were significantly higher in patients developing DGF ($n = 18$). Shortly after KTx (3–6 h), uNGAL values were higher in DGF recipients (on average +242 ng/mL, considering mean dialysis time of 4.1 years) and rose further in following days, contrasting with prompt function recipients. Day-1 uNGAL levels accurately predicted DGF (AUC-ROC = 0.93), with a performance higher than serum creatinine (AUC-ROC = 0.76), and similar to cystatin C (AUC-ROC = 0.95). Multivariable analyses revealed that uNGAL levels at days 4 and 7 were strongly associated with one-year serum creatinine. Urinary NGAL is an early marker of graft injury and is independently associated with dialysis requirement within one week after KTx and one-year graft function.

1. Introduction

Delayed graft function (DGF) is an important complication of kidney transplantation (KTx) that adversely affects allograft survival. Despite substantial improvements in the field of KTx, the incidence of DGF is rising with the growing practice of accepting expanded criteria donors to increase transplantation rates [1–6]. Delayed graft function predisposes kidney graft to acute and chronic rejection, contributes to progressive allograft dysfunction, and increases the risk of premature graft loss [7–11].

Reliable biomarkers enabling early discrimination of DGF in KTx are lacking, which impairs timely therapeutic interventions. Traditionally, acute graft dysfunction is diagnosed by measuring serum creatinine, but this parameter is an unreliable indicator of kidney function during an episode of acute injury [12]. One of the most promising biomarkers of acute kidney injury is neutrophil gelatinase-associated lipocalin (NGAL), which is released to blood from activated neutrophils during inflammatory processes. In steady situations, this lipocalin is found in urine only in trace. Massive NGAL quantities excreted in urine (uNGAL)

usually indicate damage of proximal tubular cells [13–15]. Graft injury due to ischemia-reperfusion is an inevitable consequence of KTx procedure and can result in varying degrees of early graft dysfunction, which can be considered a form of posttransplantation acute kidney injury. For this reason, several studies investigated the utility of NGAL for the diagnostic and prognostic of acute graft dysfunction following KTx [16–27]. Recently, the prognostic value of uNGAL on graft function at one-year after transplantation was also examined and presented conflicting findings [22, 28].

In order to support the usefulness of uNGAL as a reliable marker of graft injury and to clarify the role of this promising biomarker in the prediction of kidney function beyond the first week after transplant, we conducted a prospective study to

(a) evaluate longitudinal changes of uNGAL levels over the first week after KTx and identify factors associated with these changes;

(b) assess the performance of uNGAL in predicting DGF (defined as the requirement for dialysis within the first 7 days after transplantation);

(c) appraise the long-term prognostic value of uNGAL measured within one week posttransplantation on kidney allograft function, evaluated by one-year serum creatinine.

2. Subjects and Methods

Consecutive patients with end-stage renal disease, undergoing living or deceased KTx at Centro Hospitalar do Porto, from December 2010 to May 2011 were prospectively enrolled. Recruitment excluded patients less than 18 years old and those who required a combined pancreas or liver KTx. After transplant, patients with primary graft failure related to surgical causes were excluded. This study was approved by Institutional Review Board of Centro Hospitalar do Porto. Each participant provided written informed consent before enrolment.

2.1. Sample Collection and Measurements. Urine samples for NGAL determination were collected 3 to 6 h after surgery (uNGAL0 or baseline); on the following morning, nearly 8 to 12 h after graft reperfusion (uNGAL1 or first day); and then at second (uNGAL2), fourth (uNGAL4), and seventh days (uNGAL7), for a total of five samples for each patient. The same laboratory technician, who was blinded to patient information, performed uNGAL measurements using a two-step chemiluminescent microparticle immunoassay on a standardized clinical platform (ARCHITECT, Abbott Diagnostics).

Serum creatinine levels were determined preoperatively, daily until hospital discharge, and at 1, 3, 6, and 12 months after transplantation to evaluate later graft function. Serum creatinine measurements were performed by Jaffé method (Roche Diagnostics). Cystatin C was measured with a particle enhanced immunonephelometric method (Siemens Diagnostics) at the same time points as uNGAL, except for baseline.

2.2. Definitions. Delayed graft function was defined, according to United Network for Organ Sharing, as the requirement for dialysis within the first seven days after KTx, due to an absence or irrelevant improvement in graft function. Complementarily, graft function was considered "*prompt*" (non-DGF) if no dialysis was required during the first week after transplantation.

Acute Rejection was defined as either biopsy-proven rejection or antirejection treatment without biopsy.

Estimated Glomerular Filtration Rate (eGFR) was calculated using the Rule's refitted MDRD formula [29], considered to have an improved diagnostic performance and better accuracy of the true GFR in KTx recipients [30].

Creatinine Reduction Rate (%) was calculated as the difference between serum creatinine at day 2 (or day 4) and day 1, divided by serum creatinine at day 1, multiplied by 100.

Graft function at one year was evaluated by the average of the two serum creatinine levels measured closer to one year after KTx (e.g., by the average values seen at 12 and 13 months). It was thought that this would reflect more accurately the usual graft function, since a single measure could be more easily inflated by acute situations, like a urinary infection for example. Two grafts were lost at seventh and eighth months and the last serum creatinine presented by these patients prior to dialysis restart was considered as being the one-year creatinine.

2.3. Statistics. Kolmogorov-Smirnov test was performed to assess deviation from normal distribution. Quantitative variables were summarized as mean and standard deviation (SD), or as median and 25th–75th quartiles (interquartile range) for variables with skewed distribution. Categorical variables were reported as percentages.

Statistical analysis was performed in five steps. Firstly, a cross-sectional bivariate analysis was done to compare groups and study the association between uNGAL and demographic/clinical variables. Continuous variables were compared using either parametric (*t*-test) or nonparametric (Mann-Whitney) tests. Associations between categorical variables were analyzed using the χ^2 test and Fisher's exact test as appropriate. Correlations between uNGAL and continuous variables were assessed using Pearson correlation and uNGAL levels were log-transformed (ln) before analysis. Spearman correlation was used to analyze uNGAL and serum creatinine reduction ratio on posttransplant days 2 and 4.

Secondly, we used a longitudinal analysis to study uNGAL kinetics and modelling it as a response variable on time. A linear mixed-effects model was used to study the association of DGF with serial changes of uNGAL (log-transformed), controlling for donor status (living/deceased), recipient's age, time on dialysis, and time measurement of uNGAL. The interaction between DGF and uNGAL time measurement was included in the model, as such a significant interaction would suggest that DGF affects the uNGAL levels trajectory.

Thirdly, receiver-operating characteristics analysis was performed to estimate the sensitivity and specificity of uNGAL (as well as serum creatinine and cystatin C) to predict DGF. The optimal cut-off points were determined by maximizing the sum of sensitivity and specificity.

Fourthly, multivariable logistic regression analysis was undertaken to evaluate whether uNGAL levels were independently associated with DGF. Pretransplant variables known to be associated with DGF and considered potential confounders were included in the models. To avoid collinearity each time point uNGAL was included separately in different models. The final models were fitted using a backward selection procedure.

Fifthly, multivariable linear regression was used to describe the independent association of uNGAL with renal function at 12 months evaluated by serum creatinine, adjusted for the variables that usually predict graft function, including donor status, rehospitalizations, and acute rejection episodes throughout the first year. Linear regression models used log-transformed uNGAL and serum creatinine levels. As in logistic models, uNGAL at each time point were included separately in models to avoid collinearity.

All statistical analyses were done with SPSS version 20.0 and a significance level of 0.05 was considered.

3. Results

During time recruitment, 42 patients were enrolled. Two recipients had renal artery thrombosis and were excluded in the first two posttransplantation days. Therefore our study sample included 40 recipients. Baseline data are shown in Table 1.

3.1. Urinary NGAL. The first urine sample (uNGAL0) was obtained from 30 patients. On the following days, urine samples were collected from 35 patients at the first, second, and seventh days, and from 36 patients at the fourth day. All of our subjects provided at least two urine samples. Only, one patient provided merely two urine samples and the remaining 39 subjects provided 3 or more urine samples (with 20 patients providing all five samples).

Daily median uNGAL levels did not differ significantly between male and female recipients, except for the seventh day where female uNGAL levels were significantly higher. Concerning donor status, uNGAL levels were higher in deceased donor recipients at all-time points, but only statistically significant at second day. Except for the seventh day, uNGAL levels were significantly and positively correlated with cold ischemia time ($r = 0.45$, $P = 0.02$; $r = 0.36$, $P = 0.04$; $r = 0.56$, $P = 0.001$; $r = 0.46$, $P = 0.006$, resp., at baseline, first, second, and fourth days).

At most time points, uNGAL was positively and significantly correlated with recipient age ($r = 0.39$, $P = 0.02$; $r = 0.39$, $P = 0.02$; $r = 0.44$, $P = 0.007$; resp., at first, second, and seventh days) and pretransplant dialysis time ($r = 0.48$, $P = 0.008$; $r = 0.37$, $P = 0.03$; $r = 0.43$, $P = 0.01$; $r = 0.33$, $P = 0.024$; resp., at baseline, first, second, and seventh days). No significant correlation was found with HLA mismatches and with donor age and serum creatinine.

Urinary NGAL levels were significantly and positively correlated with serum creatinine at all-time points (data not shown). Furthermore, except for uNGAL0, all the remaining uNGAL levels were significantly and negatively correlated with changes in serum creatinine between the second and first days, and also between the fourth and the first days: lower uNGAL values were associated with higher reductions rates in serum creatinine (data not shown).

Median length of hospitalization after transplantation was 12 days (IQR: 7–22) and uNGAL levels were highly correlated with length of hospital stay at all-time points ($r = 0.48$, $P = 0.002$; $r = 0.64$, $P < 0.001$; $r = 0.79$, $P < 0.001$; $r = 0.77$, $P < 0.001$; $r = 0.82$, $P < 0.001$, resp., at baseline, first, second, fourth, and seventh days).

3.2. DGF and uNGAL Longitudinal Changes. Eighteen recipients (45%) had DGF, three of these were from living donors, and 22 (55%) had prompt graft function. Concerning traditional predictors of DGF and except for cold ischemia time, no significant differences were found between DGF/non-DGF in relation to baseline characteristics and induction therapy (Table 1). Mean age was significantly higher in patients with DGF (56 (11) versus 43 (16) years in non-DGF recipients, $P = 0.006$). As expected, patients with DGF had higher serum creatinine levels (Table 2) and lower creatinine reduction ratios on posttransplant days 2 and 4.

Similar to serum creatinine, median uNGAL concentrations were consistently higher in DGF group compared with non-DGF group at all measured time points (Table 2 and Figure 1). In patients with prompt graft function, the longitudinal changes of uNGAL were characterized by an initial phase with a rapid decline and then a phase with a slower decrease continuing throughout the posttransplant week. This pattern of changes was different in DGF recipients: uNGAL levels increased from baseline to the following morning after transplantation and remained elevated throughout most of the follow-up period.

A linear mixed-effects model was used to study the association of DGF with longitudinal changes of uNGAL, controlling for variables found to be associated with uNGAL by bivariate analysis. Pretransplant time on dialysis, time measurement of uNGAL, and DGF were independently associated with uNGAL levels. Adjusting for the remaining variables, donor status and recipient age lost their statistical significance and were removed from the final model (Table 3). Delayed graft function was significantly associated with uNGAL levels, with prompt function recipients having on average lower levels of uNGAL at all-time points. According to our estimation, for a patient with dialysis time of approximately 4.1 years, the initial values of uNGAL (3–6 h after transplantation) are about 242 ng/mL higher in patients who went on to develop DGF, and these values will rise even more in the following days. A significant interaction between time of measurement and DGF confirmed that longitudinal changes of uNGAL levels depend on whether the recipient had DGF or not. To clarify the meaning of this interaction,

TABLE 1: Summary of baseline and clinical characteristics in kidney transplant donors and recipients (total sample and categorized by delayed or prompt graft function).

	Total (n = 40)	DGF (n = 18)	Non-DGF (n = 22)	P value
Donor				
Age (yr)	51.2 ± 11.4	51.1 ± 13.4	51.2 ± 9.9	0.172
Male gender	26 (65)	14 (78)	12 (54.5)	0.125
Living donor	11 (27.5)	3 (16.7)	8 (36.4)	0.165
Expanded criteria donors	3 (7.5)	1 (5.6)	2 (9.1)	0.541
Serum creatinine (mg/dL)	0.81 ± 0.18	0.85 ± 0.21	0.78 ± 0.16	0.318
Donor-recipient				
HLA mismatches	3.39 ± 1.24	3.38 ± 1.07	3.41 ± 1.46	0.941
Cold ischemia time (h)	12.1 ± 7.9	15.2 ± 7.8	9.6 ± 7.3	0.035*
Living donor	2.8 ± 0.5	2.5 ± 0.5	3.0 ± 0.5	0.204
Deceased donor	16.2 ± 5.9	18.1 ± 5.1	14.1 ± 6.2	0.088
Recipient				
Age (yr)	49.2 ± 15.2	56.3 ± 10.9	43.3 ± 15.9	0.006*
Male gender	26 (65)	11 (61)	15 (68)	0.641
Caucasian	40 (100)	18 (100)	22 (100)	—
BMI (Kg/m^2)	24.8 ± 4.9	26.2 ± 4.4	23.6 ± 5.0	0.091
Previous transplant	2 (5)	0 (0)	2 (9.1)	
Time on dialysis (yr)	4.4 ± 4.7	5.6 ± 6.2	3.4 ± 2.3	0.135
Pretransplant therapy				
Dialysis	38 (95)	18 (100)	20 (90.9)	0.296
Preemptive transplantation	2 (5)	0 (0)	2 (9.1)	
Cause of kidney disease				
IgA nephropathy	7 (17.5)	2 (11.1)	5 (22.7)	—
Glomerulonephritis	6 (15.0)	4 (22.2)	2 (9.1)	—
Diabetic nephropathy	5 (12.5)	3 (16.7)	2 (9.1)	—
Autosomal dominant polycystic kidney disease	3 (7.5)	3 (16.7)	0 (0)	—
Unknown	4 (10.0)	1 (5.6)	3 (13.6)	—
Others	15 (37.5)	5 (27.8)	10 (45.5)	—
Peak PRA (%)	5.5 ± 15.1	5.0 ± 15.0	5.9 ± 15.5	0.853
0	29 (72.5)	14 (77.8)	15 (68.2)	—
1–25	8 (20.0)	3 (16.7)	5 (22.7)	—
26–75	3 (7.5)	1 (5.6)	2 (9.0)	—
Current PRA (%)	2.3 ± 8.6	3.1 ± 11.7	1.6 ± 4.9	0.585
0	34 (85)	15 (83.3)	19 (86.4)	—
1–25	5 (12.5)	2 (11.1)	3 (13.6)	—
26–50	1 (2.5)	1 (5.6)	0 (0)	—
Induction regimen				
Antithymocyte globulin (ATG-F)	4 (10)	1 (5.6)	3 (13.6)	0.613
Basiliximab/daclizumab	30 (75)	14 (77.8)	16 (72.7)	0.789
Immunosuppression at time of discharge				
Steroids	38 (95.0)	18 (100)	20 (90.9)	0.296
Tacrolimus	38 (95.0)	17 (94.4)	21 (95.5)	0.886
Cyclosporine A	2 (0.05)	1 (5.6)	1 (5.6)	0.884

Values are expressed as mean ± standard deviation or absolute numbers and percentages. Comparisons between continuous variables were done using parametric (t-test) or nonparametric (Mann-Whitney) tests; associations between categorical variables were analyzed using the χ^2 test and Fisher's exact test as appropriate; $^*P < 0.05$.
Abbreviations: HLA: human leukocyte antigen; BMI: body mass index; PRA: panel reactive antibody.

Table 2: Serial levels of serum creatinine and uNGAL through the first posttransplant week, according to graft function (delayed or prompt).

Serum Creatinine (mg/dL) Median, (IQR)	Prior transplantation	1st day* (n = 40, 18 DGF)	2nd day (n = 40, 18 DGF)	4th day (n = 40, 18 DGF)	7th day (n = 40, 18 DGF)
DGF (n = 18)	7.5 (6.0–11.7)	8.2 (6.5–9.3)	7.5 (5.9–8.5)	6.9 (6.1–8.0)	6.4 (5.3–8.9)
Non-DGF (n = 22)	7.8 (5.1–9.4)	6.3 (4.6–7.9)	4.3 (2.8–6.1)	2.5 (1.6–3.2)	1.9 (1.4–2.4)
Urine NGAL (ng/mL) Median, (IQR)	3 to 6 h after surgery (n = 30, 13 DGF)	1st day* (n = 35, 14 DGF)	2nd day (n = 35, 15 DGF)	4th day (n = 36, 15 DGF)	7th day (n = 35, 16 DGF)
DGF (n = 18)	647 (328–1648)	866 (500–1256)	834 (510–2632)	851 (549–1643)	407 (106–1249)
Non-DGF (n = 22)	256 (105–446)	129 (64–306)	80 (29–138)	47 (36–91)	34 (26–57)

*1st day = 8 to 12 h after surgery; values are medians and interquartile range (25th to 75th percentile).
Abbreviations: uNGAL: urinary neutrophil gelatinase associated lipocalin; IQR: interquartile range; DGF: delayed graft function; non-DGF: prompt function.

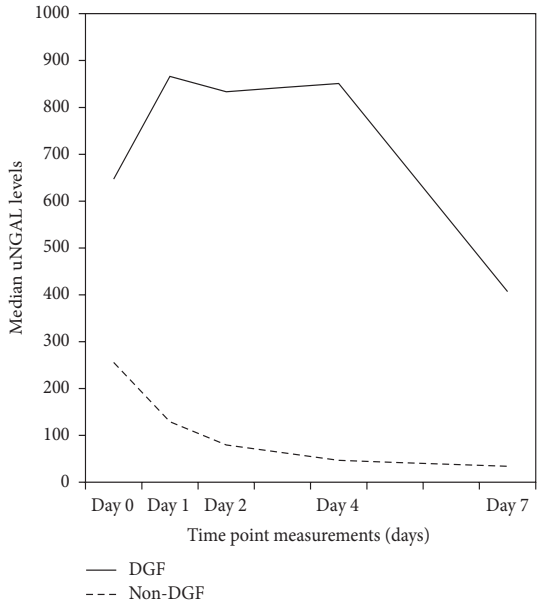


Figure 1: Evolution of uNGAL levels through first week after transplantation, according to graft function. Abbreviations: uNGAL: urinary neutrophil gelatinase associated lipocalin; DGF: delayed graft function.

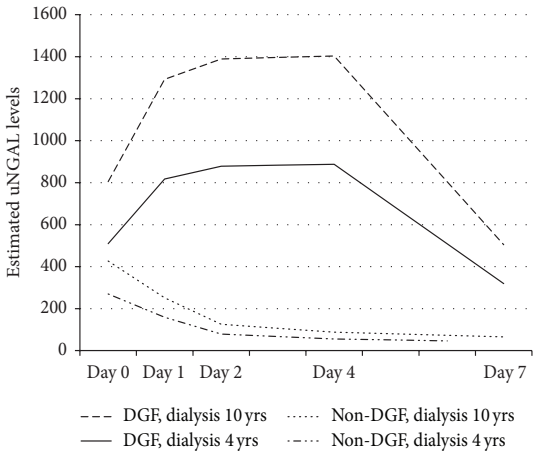


Figure 2: Predicted uNGAL values over time of four hypothetical subjects, estimated from multiple linear mixed model presented in Table 3. Abbreviations: uNGAL: urinary neutrophil gelatinase associated lipocalin; DGF: delayed graft function; non-DGF: prompt graft function.

Figure 2 shows the predicted uNGAL trajectories over time for four hypothetical subjects: two recipients who developed DGF (one with 4 years of dialysis and one with 10 years), and two other patients with prompt graft function (similar time on dialysis, 4 and 10 years). Hypothetically, the remaining variables were equal in all four patients. The predicted uNGAL values were estimated using the coefficients estimates of Table 3 (e.g., the predicted uNGAL values at the first day for a recipient with 4 years of dialysis with prompt function = exp [(5.46 − 2.14) + 0.94 + 0.4 + (0.076 ∗ 4 years of dialysis)] = 158 ng/mL).

3.3. Prediction of DGF by uNGAL Levels (ROC analysis). Receiver-operating characteristic (ROC) curves showed uNGAL on the first postoperative days were accurate in predicting DGF (Tables 4 and 5). Table 4 displays the derived sensitivities, specificities, and predictive values for uNGAL at the cutoff concentrations that provided the maximum sum of sensitivity and specificity. Regarding the areas under the ROC curves (AUC), the ability of uNGAL to predict DGF was moderately accurate at baseline and first day, and highly accurate at second, fourth, and seventh days (Table 5, Figure 3). In the first two posttransplant days the diagnostic performance of uNGAL was better than of serum creatinine, and quite similar to that of cystatin C. The reduction in serum creatinine between first and second days resulted in AUC = 0.78 [0.64–0.92] and was worse than uNGAL for DGF prediction.

Table 3: Results of the final linear mixed model for dependent variable ln(uNGAL) (n = 171 observations derived from 40 patients).

	Coefficient estimate	P value	95% CI	
Intercept	5.46	<0.001	4.94	5.98
Graft function				
DGF = 0 (prompt graft function)	−2.04	<0.001	−2.64	−1.44
DGF = 1 (with DGF-reference)	0	—	—	—
Time				
Time (3 to 6 h after surgery)	0.47	0.088	−0.07	1.00
Time (1st day)	0.94	0.001	0.41	1.47
Time (2nd day)	1.01	<0.001	0.49	1.53
Time (4th day)	1.02	<0.001	0.50	1.54
Time (7th day-reference)	0	—	—	—
Time ∗ DGF				
Time (3 to 6 h after surgery) ∗ DGF = 0	1.40	<0.001	0.68	2.13
Time (1st day) ∗ DGF = 0	0.40	0.257	−0.29	1.10
Time (2nd day) ∗ DGF = 0	−0.37	0.295	−1.06	0.32
Time (4th day) ∗ DGF = 0	−0.73	0.039	−1.42	−0.03
Time (7th day) ∗ DGF = 0 (reference)	0	—	—	—
Time on dialysis	0.076	0.003	0.03	0.12

Abbreviations: uNGAL: urinary neutrophil gelatinase associated lipocalin; ln: natural logarithm; DGF: delayed graft function (DGF = 0, no delayed graft function).

Table 4: Sensitivity, specificity, and predictive values for DGF using specific uNGAL cut-off values.

Time after transplant	uNGAL cutoff (ng/mL)	Sensitivity (%)	Specificity (%)	PPV	NPV
Shortly after surgery (3 to 6 h)	479	77	88	87	79
1st day (8 to 12 h after surgery)	286	100	76	81	100
2nd day	277	93	90	90	93
4th day	232	93	95	95	93
7th day	63	94	84	86	93

DGF: delayed graft function; uNGAL: urinary neutrophil gelatinase-associated lipocalin; PPV: positive predictive value; NPV: negative predictive value.

3.4. Independent Association of uNGAL Levels and DGF (Multivariable Analyses). Multivariable logistic regression analyses revealed that uNGAL levels remained independently associated with DGF at most time points, after adjusting for clinically relevant risk factors for DGF (Table 6). Furthermore, recipient age was the other significant independent predictor of DGF in almost all models. To be more clinically relevant, estimates of DGF risk were converted to every 50 ng/mL of increase in uNGAL or per each 5 years of increase in age, instead of estimates per each unit of increase.

3.5. Within One-Year after Kidney Transplantation. During the first year, 10 KTx recipients were rehospitalized accounting for a total of 19 hospital admissions. There was one rehospitalization in six patients, two in two patients, three in one patient, and six rehospitalizations in one patient with a psychological disorder and suicidal ideation. The causes of rehospitalization were infection in five admissions (mostly, urinary tract infection), renal dysfunction in six, and nonrenal causes in the remaining eight admissions (suicidal ideation, acute pulmonary edema, and neutropenia).

Excluding the recipient with several rehospitalizations due to psychological decompensation, the length of hospital stay of the remaining recipients admissions was 7 [3] days, and no significant differences were found between recipients from living or deceased donors.

The acute rejection episodes were collected throughout the first posttransplant year. Ten recipients (25%) had an acute rejection episode during inpatient hospitalization for transplant surgery, and only one patient was rehospitalized one month after KTx with an acute rejection episode confirmed by biopsy.

At one year after transplantation, all patients were alive but two grafts were lost. At this time, the median plasma creatinine was significantly higher in DGF group compared to non-DGF: 1.6 mg/dL [IQR: 1.2–2.5] versus 1.3 mg/dL [IQR: 1.0–1.5], P = 0.049.

3.6. Prognostic Value of First-Week uNGAL Levels in One-Year Graft Function. The correlation between uNGAL collected in the first week after KTx and serum creatinine at one year was explored. Except for uNGAL collected within the first 24 h after transplantation, uNGAL levels were positively correlated with serum creatinine evaluated at time of discharge, and also at 1, 3, 6, and 12 months. Likewise, uNGAL levels at

Table 5: Area under the receiver-operating characteristic curve at each time point for uNGAL, serum creatinine, and serum cystatin C for predicting DGF.

	Time after transplant	AUC (95% CI)	*P* value
Urine NGAL (ng/mL)	Shortly after surgery (3 to 6 h)	0.77 (0.58–0.97)	0.010
	1st day (8 to 12 h after surgery)	0.88 (0.77–1.0)	<0.001
	2nd day	0.96 (0.90–1.0)	<0.001
	4th day	0.99 (0.97–1.0)	<0.001
	7th day	0.93 (0.86–1.0)	<0.001
Serum creatinine (mg/dL)	Prior transplantation	0.56 (0.38–0.74)	0.514
	1st day (8 to 12 h after surgery)	0.77 (0.61–0.93)	0.007
	2nd day	0.90 (0.79–1.0)	<0.001
	4th day	0.95 (0.87–1.0)	<0.001
	7th day	0.93 (0.81–1.0)	<0.001
Serum cystatin C (mg/L)	1st day (6 to 12 h after surgery)	0.90 (0.79–1.0)	<0.001
	2nd day	0.96 (0.88–1.0)	<0.001
	4th day	0.95 (0.89–1.0)	<0.001
	7th day	0.93 (0.83–1.0)	<0.001

DGF: delayed graft function; uNGAL: urinary neutrophil gelatinase-associated lipocalin.

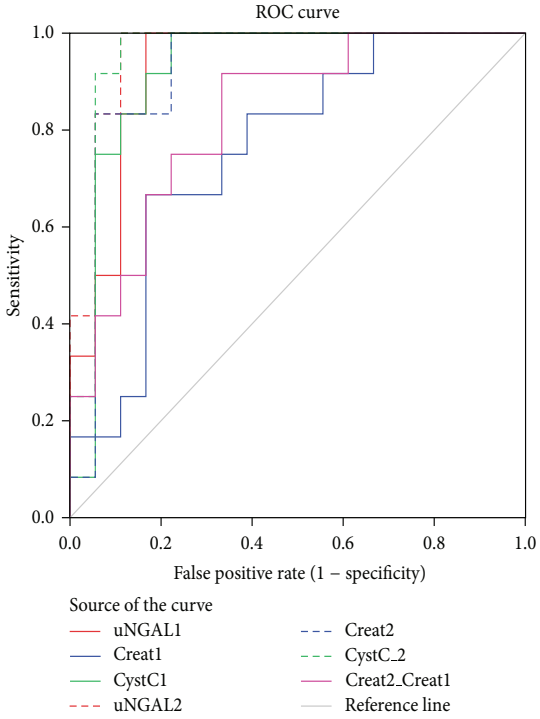


Figure 3: Receiver-operating characteristic curves for uNGAL, serum creatinine and changes in serum creatinine, and serum cystatin C measured at posttransplant days 1 and 2 for predicting delayed graft function. Abbreviations: uNGAL: urinary neutrophil gelatinase associated lipocalin; Creat: serum creatinine; Cyst: serum Cystatin C; Creat2-Creat1: creatinine reduction rate between the first and the second day.

days 2, 4, and 7 were inversely correlated with eGFR at 6 and 12 months (data not shown).

The prognostic value of early uNGAL values on long-term allograft function (one year after KTx) was tested by multivariable analysis. In multivariable linear regression models for serum creatinine at 12 months, uNGAL measured on the fourth and seventh days were independently associated with one-year graft function, adjusting for established variables that usually affect graft function, including acute rejection episodes and rehospitalizations that occurred during the first posttransplant year (Table 7).

4. Discussion

The major finding of this study is that uNGAL is a promising biomarker for allograft dysfunction that can be easily and noninvasively assayed in the early posttransplant period. We prospectively evaluated uNGAL in a cohort of 40 kidney allograft recipients during the first posttransplant week. At all measured time points, uNGAL levels were consistently higher in patients who developed DGF, including the earliest levels obtained from the first urine sample collected approximately 3 to 6 h after transplant surgery. At this time, clinical diagnosis of DGF is yet not possible, but a simple and noninvasive test can already recognize kidney dysfunction and stratify patients according to likelihood of requiring posttransplant dialysis.

It would be ideal to diagnose graft dysfunction with an early and highly sensitive biologic marker of renal tubular injury. One of the most promising markers is NGAL, and our findings provide further information for the use of uNGAL as a diagnostic and prognostic tool for DGF. According to our estimation, uNGAL values shortly after transplant surgery will be much higher in patients who went on to develop DGF and will rise further in the following days. In contrast, patients with prompt function will have lower levels, which decrease consistently along the week. The kinetics of changes in these recipients compared to those who presented DGF is quite different. It seems that, not only the baseline levels, but also the pattern of uNGAL longitudinal changes can reflect graft dysfunction.

The association between higher NGAL levels and DGF after KTx has been previously published [16, 20, 22, 24, 25]. But the findings are not consistent regarding the kinetics of uNGAL according to DGF. Hollmen et al. [22] found initial levels of uNGAL higher in DGF patients, but on the following day a decrease was observed, as it happened with recipients with prompt function. As mentioned before, our study did not confirm this declining in DGF patients. Recipients who went on to develop DGF had initial higher levels of uNGAL that rise further on the following posttransplant days, differing from patients with prompt graft function. Our findings are in agreement with results reported by Hall

Table 6: Association of uNGAL with delayed graft function by multivariable analysis (logistic regression).

	Delayed graft function		
	OR adjusted*	*P* value	95% CI
Model 1 (uNGAL at 3 to 8 h after surgery)			
uNGAL0 (per 50 ng/mL of increase)	1.15	0.044	1.01–1.31
Recipient age (per 5 years of increase)	1.49	0.054	0.99–2.24
Model 2 with (uNGAL at day 1)			
uNGAL1 (per each 50 ng/mL of increase)	1.22	0.012	1.05–1.42
Recipient age (per 5 years of increase)	1.99	0.022	1.11–3.57
Model 3 (uNGAL at day 2)			
uNGAL2 (per each 50 ng/mL of increase)	1.35	0.004	1.10–1.66
Model 4 (uNGAL at day 4)			
uNGAL4 (1 ng/mL increase)	3.01	0.035	1.08–8.40
Model 5 (uNGAL at day 7)			
uNGAL7 (per each 50 ng/mL of increase)	1.43	0.050	1.01–2.04
Recipient age (per 5 years of increase)	1.73	0.038	1.03–2.90

Note: results given by logistic regression (backward Wald test).
Abbreviations: OR: odds ratio; 95% CI (95% confidence interval); uNGAL: urinary neutrophil gelatinase-associated lipocalin.
* Adjusted for pretransplant time on dialysis, recipient gender and age, and donor age.

Table 7: Significant factors associated with serum creatinine at one year after kidney transplantation.

	Regression coefficient adjusted*	*P*-value	95% CI
Model with uNGAL at day 4			
Donor gender (male versus female)	0.042	0.004	0.015–0.069
Donor age (years)	0.011	0.008	0.003–0.020
uNGAL4 (ln, ng/mL)	0.067	0.045	0.002–0.132
Model with uNGAL at day 7			
Time on dialysis (ln, ng/mL)	0.042	0.004	0.015–0.069
Donor age (years)	0.018	0.002	0.008–0.029
uNGAL7 (ln, ng/mL)	0.138	0.007	0.041–0.235

Note: results given by multiple linear regression; serum creatinine (ln) at 12 months as the dependent variable. Only the significant variables associated with serum creatinine are displayed. Abbreviations: uNGAL: urinary neutrophil gelatinase-associated lipocalin.
* Adjusted for donor status, donor age, recipient age and gender, pretransplant time on dialysis, rehospitalizations, and acute rejection episodes throughout the first year.

et al. [24]. It seems that, above and beyond the markedly higher levels of uNGAL in patients with graft dysfunction, the contrasting pattern of uNGAL longitudinal changes can distinguish recipients who will need dialysis in the first week posttransplantation.

To the best of our knowledge, this is the first report that used linear mixed analysis in describing longitudinal changes of uNGAL in the first week following KTx. Multiple observations of a variable on a particular patient are likely to be positively correlated, so they should not be treated as independent measurements. Although models that take this design into consideration are more complicated, they are also more specific and powerful since they permit the study of changes over time. Linear mixed analysis not only permits to model individual changes over time, but also is able to distinguish within-subject from between-subject sources of variation [31].

In accordance with previously published data [16, 20–22, 24], we confirmed the good performance of NGAL in predicting graft dysfunction in the early posttransplant period. Using ROC analysis, our study also corroborates uNGAL as a good diagnostic marker on identifying patients with graft dysfunction and who subsequently required dialysis. The AUC-ROC for uNGAL was moderately accurate for DGF prediction within the first day after transplant, and it was excellent at day 2 and day 4. We also determined the paired sensitivity and specificity for the cutoff value of uNGAL, calculated to be closest to the left upper corner of the ROC space to predict DGF. At 8 to 12 h after surgery, a cutoff of 286 ng/mL had 100% sensitivity and 76% specificity for the identification of DGF. Within the second day, uNGAL levels higher than 277 ng/mL predicted DGF with a sensitivity of 93% and specificity of 90%. Other studies showed also impressive results. Parikh et al. [16] in a study that included 53 patients undergoing KTx, measured NGAL in urine samples collected within the first 24 h following transplantation and reported an AUC-ROC of 0.9, similar to ours obtained 8 to 12 h after surgery. Another study [24] conducted in 91 recipients evaluated uNGAL within 6 h after transplantation and predicted subsequent DGF with an AUC-ROC = 0.81.

Most recently, Hollmen et al. [22] undertook a large cohort study that included 176 KTx recipients. Urine was collected before transplant, at then at days 1, 3, 7, and 14, and uNGAL was measured at each time point. The authors found and AUC-ROC = 0.74 at day 1.

We report a superior performance of uNGAL level for predicting DGF over serum creatinine measured at the same time. Urinary NGAL measured at the first day predicted DGF with an AUC-ROC of 0.93, which is markedly better than an AUC-ROC = 0.76 shown by serum creatinine measured in the same day, and also than an AUC-ROC = 0.83 obtained from creatinine reduction ratio from first to second day, but quite similar to cystatin C (0.95), a marker considered more accurately to detect changes in renal function [32–35]. Furthermore, our analyses also revealed that uNGAL levels predicted DGF, even after adjusting for pretransplant variables known to be traditionally associated with DGF.

Besides DGF, the other factors that significantly influenced uNGAL levels were previous time on dialysis, recipient's age at time of transplantation and cold ischemia time. These three variables were positively correlated with uNGAL values. Mishra and coworkers [17] have shown that the immunohistochemical staining intensity for NGAL was strongly correlated with cold ischemia time and NGAL expression was significantly increased in deceased donor biopsies. We found that uNGAL levels were higher in graft recipients from deceased donors, but only significantly higher at the second day. It is known that prolonged cold preservation of kidneys can lead to severe injury, which is critical in the success of deceased-donor kidney transplantation [36]. However, there is a progressive effort of our transplant team to avoid prolonged cold preservation. Maybe this attempt attenuated the effect of cold ischemic injury in kidneys from deceased donors, which become comparable to living donors concerning uNGAL values. An interesting finding of our study was that uNGAL levels at all-time points were correlated with length of hospital stay. It is well known that the occurrence of DGF prolongs the recipient's hospital stay. And it is worthy of note to realize that patients with early higher levels of uNGAL will expect longer time of hospitalization, probably due to graft dysfunction.

As other studies [16–19, 24, 26, 37], we confirmed that uNGAL levels were inversely correlated with eGFR and positively correlated with serum creatinine at each measured time point. We also showed that not only in the first week, but longer after that, uNGAL levels measured in the first seven days after KTx were still predictive of graft function throughout the first year after transplantation. Even after adjusting for donor status, acute rejection episodes, hospitalizations occurred in the first year, and other known variables that usually affect graft function, uNGAL evaluated at days four and seven were predictive of one-year serum creatinine, which can be considered a surrogate marker of long-term graft survival [38, 39]. In contrast, Hollmen and coworkers study [22] did not find any correlation between uNGAL and renal function at one year. In their study, uNGAL collected in the first two weeks after transplantation was only correlated with renal function up to 3 months. Our results do not corroborate this lack of correlation and are in agreement with a recent study that also associated perioperative uNGAL levels to one-year allograft function [28].

Our study has several strengths. First, it is a prospective-cohort design study. Second, we measured uNGAL at several time points within the first posttransplant week, and not at one single point. Longitudinal studies have the advantage of providing detailed information about how a marker changes over time; however the studies present some statistical complexities, since the customary assumption that all observations are independent usually does not hold. And this was the third strength of our study: the use of longitudinal methods to handle the serial changes of uNGAL. A fourth strength was the technical determination that we have chosen to measure NGAL. We used a commercially available kit for uNGAL determination (Abbott Architect NGAL), which is simple to implement in routine practice and it is considered one of the best methods for detecting acute kidney injury [40].

Similarly to other authors [16, 22], we have chosen to measure NGAL in urine, instead of blood, since uNGAL represents tubule damage in the kidney rather than filtration from blood [14, 41]. An increased level of NGAL in urine usually indicates injury of proximal tubular cells and seems to be more specific compared to serum NGAL, which can be produced by other organs and released into the circulation following a transplant surgery [42]. Other advantages of urinary diagnostics include the noninvasive nature of sample collection and the reduced number of interfering protein [43]. However, despite the undoubtedly value of urinary markers of kidney injury, their use in transplant recipients can be also a drawback because of possible transient graft anuria, which may preclude the availability of urine and consequently the lack of sample to measure NGAL. Due to the shortcoming of urine biomarkers in anuric KTx recipients, some studies have evaluated the performance of serum/plasma NGAL in predicting graft function recovery after KTx [21, 27]. As we did not measure serum/plasma NGAL values, we could not compare their effectiveness in predicting DGF in our sample. In our study, 4 or 5 recipients were anuric in each measurement, resulting in 12% of our patients not having urine sample to determine uNGAL in that particular time point. The measurement of serum/plasma NGAL could have been a valuable alternative in these recipients, since it could also be obtained noninvasively in patients who required dialysis during the transient period of anuria.

Similar to other areas in medicine, in kidney transplantation early diagnosis and timely intervention will improve outcomes. Ischemic injury of the renal allograft is a critical early insult that increases the risk of acute tubular necrosis and long-term graft loss. If DGF could be detected in the early hours after surgical procedure, maybe a tailored and more individualized intervention could be achieved. Perioperative fluid management must ensure the restoration and maintenance of the intravascular volume, in order to obtain an appropriate graft function. Aggressive hydration has been recognized to be effective in avoiding DGF, but fluid overload may also precipice the need of dialysis with the risk of hipovolemia and consequent renal ischemia. Early identification of DGF patients could allow to be more

judicious and to modify postoperative fluid management in favor of maintaining just adequate filling pressures to maintain adequate intravascular volume and prevent fluid overload [44]. Regarding immunosuppression, the induction protocol chosen for this group of patients should have the associated effect of decreasing DGF rates, by suppressing leukocyte-rich vascular congestion and endothelial injury, and the introduction of calcineurin inhibitors could be avoided or delayed due to their vasoconstrictive properties. Cytomegalovirus (CMV) infection has also direct and indirect effects on transplant graft function, and some previous evidence has been published relating the association between the use of ganciclovir and the lower occurrence of DGF [45]. Nowadays, the prophylaxis with valganciclovir should be other aspect taken into account in recipients that we know they will develop DGF, since this prophylaxis may do more than just delay the occurrence of CMV disease.

Several studies were done in renal transplantation to identify early biomarkers for the diagnosis of DGF. However, there is still no routine application of any of these markers in clinical transplantation. The present study clearly support that uNGAL represents an early marker of graft injury and is strongly associated with dialysis-based diagnosis of DGF and one-year graft function. Other studies are necessary to clarify the genesis and sources of plasma and urinary NGAL and validate the accuracy of uNGAL as a diagnostic marker of renal graft injury and predictor for DGF in assorted centres, across different practices and sets of variables.

Conflict of Interests

The authors declare that there is no conflict regarding the publication of this paper. The results presented in this paper have not been published previously in whole or part, neither in abstract format.

Authors' Contribution

Isabel Fonseca designed the study, collected data, compiled the database, performed statistical analyses, interpreted the data, and wrote the paper. José Carlos Oliveira and Madalena Cruz were responsible for the assays. Manuela Almeida, Anabela Malho, La Salete Martins, Sofia Pedroso, Leonídio Dias, Josefina Santos, and António Castro Henriques were involved in patients recruiting patients and coordinated the collection of samples and assembling the patient information. António Castro Henriques and Denisa Mendonça helped to design and coordinate the study. José Carlos Oliveira, Luísa Lobato, António Castro Henriques, and Denisa Mendonça revised the drafts and gave helpful comments for analysis and interpretation of the data. All authors approved the final version.

Acknowledgments

The authors recognize and thank Abbott Laboratories for their valuable contribution for donating kits used for testing almost 200 samples. The remaining kits were financed by funds of Unit for Multidisciplinary Investigation in Biomedicine, Porto, Portugal. The investigators also thank Dr. António Cabrita for allowing the study, and especially to nursing and laboratory staff for their commitment and crucial help on samples collection.

References

[1] N. Perico, D. Cattaneo, M. H. Sayegh, and G. Remuzzi, "Delayed graft function in kidney transplantation," *Lancet*, vol. 364, no. 9447, pp. 1814–1827, 2004.

[2] A. O. Ojo, R. A. Wolfe, P. J. Held, F. K. Port, and R. L. Schmouder, "Delayed graft function: risk factors and implications for renal allograft survival," *Transplantation*, vol. 63, no. 7, pp. 968–974, 1997.

[3] O. H. J. Koning, J. H. van Bockel, F. J. van der Woude, G. G. Persijn, J. Hermans, and R. J. Ploeg, "Risk factors for delayed graft function in University of Wisconsin solution preserved kidneys from multiorgan donors," *Transplantation Proceedings*, vol. 27, no. 1, pp. 752–753, 1995.

[4] M. T. Sellers, M. H. Gallichio, S. L. Hudson et al., "Improved outcomes in cadaveric renal allografts with pulsatile preservation," *Clinical Transplantation*, vol. 14, no. 6, pp. 543–549, 2000.

[5] D. W. Gjertson, "Impact of delayed graft function and acute rejection on kidney graft survival," *Clinical Transplants*, pp. 467–480, 2000.

[6] S. G. Yarlagadda, S. G. Coca, A. X. Garg et al., "Marked variation in the definition and diagnosis of delayed graft function: a systematic review," *Nephrology Dialysis Transplantation*, vol. 23, no. 9, pp. 2995–3003, 2008.

[7] H. Boom, M. J. K. Mallat, J. W. De Fijter, A. H. Zwinderman, and L. C. Paul, "Delayed graft function influences renal function, but not survival," *Kidney International*, vol. 58, no. 2, pp. 859–866, 2000.

[8] M. Giral-Classe, M. Hourmant, D. Cantarovich et al., "Delayed graft function of more than six days strongly decreases long-term survival of transplanted kidneys," *Kidney International*, vol. 54, no. 3, pp. 972–978, 1998.

[9] C. Y. Lu, J. G. Penfield, M. L. Kielar, M. A. Vazquez, and D. R. Jeyarajah, "Hypothesis: is renal allograft rejection initiated by the response to injury sustained during the transplant process," *Kidney International*, vol. 55, no. 6, pp. 2157–2168, 1999.

[10] D. A. Shoskes and J. M. Cecka, "Deleterious effects of delayed graft function in cadaveric renal transplant recipients independent of acute rejection," *Transplantation*, vol. 66, no. 12, pp. 1697–1701, 1998.

[11] C. Troppmann, K. J. Gillingham, R. W. G. Gruessner et al., "Delayed graft function in the absence of rejection has no long-term impact. A study of cadaver kidney recipients with good function at 1 year after transplantation," *Transplantation*, vol. 61, no. 9, pp. 1331–1337, 1996.

[12] R. L. Mehta and G. M. Chertow, "Acute renal failure definitions and classification: time for change?" *Journal of the American Society of Nephrology*, vol. 14, no. 8, pp. 2178–2187, 2003.

[13] D. Bolignano, G. Coppolino, A. Lacquaniti, and M. Buemi, "From kidney to cardiovascular diseases: NGAL as a biomarker beyond the confines of nephrology," *European Journal of Clinical Investigation*, vol. 40, no. 3, pp. 273–276, 2010.

[14] K. Mori, H. T. Lee, D. Rapoport et al., "Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from

ischemia-reperfusion injury," *Journal of Clinical Investigation*, vol. 115, no. 3, pp. 610–621, 2005.

[15] J. Mishra, M. A. Qing, A. Prada et al., "Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury," *Journal of the American Society of Nephrology*, vol. 14, no. 10, pp. 2534–2543, 2003.

[16] C. R. Parikh, A. Jani, J. Mishra et al., "Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation," *American Journal of Transplantation*, vol. 6, no. 7, pp. 1639–1645, 2006.

[17] J. Mishra, Q. Ma, C. Kelly et al., "Kidney NGAL is a novel early marker of acute injury following transplantation," *Pediatric Nephrology*, vol. 21, no. 6, pp. 856–863, 2006.

[18] J. Malyszko, J. S. Malyszko, and M. Mysliwiec, "Serum neutrophil gelatinase-associated lipocalin correlates with kidney function in renal allograft recipients," *Clinical Transplantation*, vol. 23, no. 5, pp. 681–686, 2009.

[19] J. Malyszko, J. S. Malyszko, H. Bachorzewska-Gajewska, B. Poniatowski, S. Dobrzycki, and M. Mysliwiec, "Neutrophil gelatinase-associated lipocalin is a new and sensitive marker of kidney function in chronic kidney disease patients and renal allograft recipients," *Transplantation Proceedings*, vol. 41, no. 1, pp. 158–161, 2009.

[20] U. Lebkowska, J. Malyszko, A. Lebkowska et al., "Neutrophil gelatinase-associated lipocalin and cystatin C could predict renal outcome in patients undergoing kidney allograft transplantation: a prospective study," *Transplantation Proceedings*, vol. 41, no. 1, pp. 154–157, 2009.

[21] M. Kusaka, Y. Kuroyanagi, T. Mori et al., "Serum neutrophil gelatinase-associated lipocalin as a predictor of organ recovery from delayed graft function after kidney transplantation from donors after cardiac death," *Cell Transplantation*, vol. 17, no. 1-2, pp. 129–134, 2008.

[22] M. E. Hollmen, L. E. Kyllönen, K. A. Inkinen, M. L. T. Lalla, and K. T. Salmela, "Urine neutrophil gelatinase-associated lipocalin is a marker of graft recovery after kidney transplantation," *Kidney International*, vol. 79, no. 1, pp. 89–98, 2011.

[23] M. E. Hollmen, L. E. Kyllönen, K. A. Inkinen, M. L. T. Lalla, J. Merenmies, and K. T. Salmela, "Deceased donor neutrophil gelatinase-associated lipocalin and delayed graft function after kidney transplantation: a prospective study," *Critical Care*, vol. 15, no. 3, article R121, 2011.

[24] I. E. Hall, S. G. Yarlagadda, S. G. Coca et al., "IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation," *Journal of the American Society of Nephrology*, vol. 21, no. 1, pp. 189–197, 2010.

[25] A. Bataille, S. Abbas, O. Semoun et al., "Plasma neutrophil gelatinase-associated lipocalin in kidney transplantation and early renal function prediction," *Transplantation*, vol. 92, no. 9, pp. 1024–1030, 2011.

[26] N. E. Magnusson, M. Hornum, K. A. Jorgensen et al., "Plasma neutrophil gelatinase associated lipocalin (NGAL) is associated with kidney function in uraemic patients before and after kidney transplantation," *BMC Nephrology*, vol. 13, article 8, 2012.

[27] M. Kusaka, F. Iwamatsu, Y. Kuroyanagi et al., "Serum neutrophil gelatinase associated lipocalin during the early postoperative period predicts the recovery of graft function after kidney transplantation from donors after cardiac death," *Journal of Urology*, vol. 187, no. 6, pp. 2261–2267, 2012.

[28] I. E. Hall, M. D. Doshi, P. P. Reese, R. J. Marcus, H. Thiessen-Philbrook, and C. R. Parikh, "Association between peritransplant kidney injury biomarkers and 1-year allograft outcomes," *Clinical Journal of the American Society of Nephrology*, vol. 7, no. 8, pp. 1224–1233, 2012.

[29] A. D. Rule, T. S. Larson, E. J. Bergstralh, J. M. Slezak, S. J. Jacobsen, and F. G. Cosio, "Using serum creatinine to estimate glomerular filtration rate: accuracy in good health and in chronic kidney disease," *Annals of Internal Medicine*, vol. 141, no. 12, pp. 929–937, 2004.

[30] U. Pöge, T. Gerhardt, B. Stoffel-Wagner et al., "Can modifications of the MDRD formula improve the estimation of glomerular filtration rate in renal allograft recipients?" *Nephrology Dialysis Transplantation*, vol. 22, no. 12, pp. 3610–3615, 2007.

[31] G. M. Fitzmaurice and C. Ravichandran, "A primer in longitudinal data analysis," *Circulation*, vol. 118, no. 19, pp. 2005–2010, 2008.

[32] E. Premaratne, R. J. MacIsaac, S. Finch, S. Panagiotopoulos, E. Ekinci, and G. Jerums, "Serial measurements of cystatin c are more accurate than creatinine-based methods in detecting declining renal function in type 1 diabetes," *Diabetes Care*, vol. 31, no. 5, pp. 971–973, 2008.

[33] L. A. Stevens, J. Coresh, C. H. Schmid et al., "Estimating GFR using serum cystatin C alone and in Combination with serum creatinine: a pooled analysis of 3,418 individuals with CKD," *American Journal of Kidney Diseases*, vol. 51, no. 3, pp. 395–406, 2008.

[34] B. A. Perkins, R. G. Nelson, B. E. P. Ostrander et al., "Detection of renal function decline in patients with diabetes and normal or elevated GFR by serial measurements of serum cystatin C concentration: results of a 4-year follow-up study," *Journal of the American Society of Nephrology*, vol. 16, no. 5, pp. 1404–1412, 2005.

[35] O. F. Laterza, C. P. Price, and M. G. Scott, "Cystatin C: an improved estimator of glomerular filtration rate?" *Clinical Chemistry*, vol. 48, no. 5, pp. 699–707, 2002.

[36] A. K. Salahudeen and W. May, "Reduction in cold ischemia time of renal allografts in the United States over the last decade," *Transplantation Proceedings*, vol. 40, no. 5, pp. 1285–1289, 2008.

[37] M. Szewczyk, T. Wielkoszyński, M. Zakliczyński, and M. Zembala, "Plasma neutrophil gelatinase-associated lipocalin (NGAL) correlations with cystatin C, serum creatinine, and glomerular filtration rate in patients after heart and lung transplantation," *Transplantation Proceedings*, vol. 41, no. 8, pp. 3242–3243, 2009.

[38] I. Fonseca, M. Almeida, L. S. Martins et al., "First-year renal function predicts long-term renal allograft loss," *Transplantation Proceedings*, vol. 43, no. 1, pp. 106–112, 2011.

[39] S. Hariharan, M. A. McBride, W. S. Cherikh, C. B. Tolleris, B. A. Bresnahan, and C. P. Johnson, "Post-transplant renal function in the first year predicts long-term kidney transplant survival," *Kidney International*, vol. 62, no. 1, pp. 311–318, 2002.

[40] E. Cavalier, A. C. Bekaert, A. Carlisi, D. Legrand, J. M. Krzesinski, and P. Delanaye, "Neutrophil gelatinase-associated lipocalin (NGAL) determined in urine with the Abbott architect or in plasma with the Biosite Triage? The laboratory's point of view," *Clinical Chemistry and Laboratory Medicine*, vol. 49, no. 2, pp. 339–341, 2011.

[41] T. Kuwabara, K. Mori, M. Mukoyama et al., "Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons," *Kidney International*, vol. 75, no. 3, pp. 285–294, 2009.

[42] J. B. Cowland, O. E. Sørensen, M. Sehested, and N. Borregaard, "Neutrophil gelatinase-associated lipocalin is up-regulated in

human epithelial cells by IL-1 beta, but not by TNF-α," *Journal of Immunology*, vol. 171, no. 12, pp. 6630–6639, 2003.

[43] P. Devarajan, "Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury," *Biomarkers in Medicine*, vol. 4, no. 2, pp. 265–280, 2010.

[44] P. Schnuelle and F. Johannes van der Woude, "Perioperative fluid management in renal transplantation: a narrative review of the literature," *Transplant International*, vol. 19, no. 12, pp. 947–959, 2006.

[45] J. A. Fishman, V. Emery, R. Freeman et al., "Cytomegalovirus in transplantation—challenging the status quo," *Clinical Transplantation*, vol. 21, no. 2, pp. 149–158, 2007.

4.3. STUDY III

OXIDATIVE STRESS IN KIDNEY TRANSPLANTATION

Fonseca I, Reguengo H, Almeida M, Dias L, Martins LS, Pedroso S, Santos J, Lobato L, Henriques AC, Mendonça D. Oxidative Stress in Kidney Transplantation: Malondialdehyde is an early predictive marker of graft dysfunction. *Transplantation* 2014; 97: 1058-65.

Ischemia-reperfusion injury is an inevitable phenomena of kidney transplantation and oxidative stress is a significant component of this process. Oxygen free radicals and impaired antioxidant activity are some of the most likely agents responsible for initiating the damage associated with reperfusion injury in kidney transplantation. Given this, and following the same methodology of the study II, this research was designed to evaluate longitudinal changes of a lipid peroxidation marker (MDA) and some antioxidant parameters (TAS, SOD, GPx, and GR) in the first week after kidney transplantation and to identify the factors that are associated with these changes as well as investigate their accuracy in predicting DGF and one-year graft function as evaluated by SCr.

The main results are described below:

- After transplantation, the mean MDA levels were consistently higher in DGF patients at all timepoints than in non-DGF recipients, but no differences were found in relation to the antioxidant parameters.

- Using a linear mixed-effects model to analyze the longitudinal changes of MDA by the two groups of patients (DGF/ non-DGF) by controlling for variables that were found to be associated with MDA levels by bivariate analysis (donor status and recipient age), we confirmed the independent association of DGF with the MDA levels. The donor status and recipient age lost their statistical significance and were removed from the final model. The time measurement of MDA and DGF were the only independent factors associated with the MDA levels, and recipients who had prompt function had, on average, lower levels of MDA at all timepoints. According to our estimation, the first values of MDA after transplantation were 0.16 µmol/L higher in patients who went on to develop DGF. A significant interaction between the time of measurement and DGF confirmed that longitudinal changes in the MDA levels depend on whether the recipient developed DGF or not.

- The effect of DGF on the progression of antioxidant parameters over time was not statistically significant.

- Receiver-operating characteristic analyses were performed to assess the potential of oxidative markers to predict DGF. Only the MDA levels at all post-transplant timepoints predicted the need for dialysis within the first week. The level of MDA at day-1 was a very good predictor for the early diagnosis of DGF (AUC=0.90), and its diagnostic accuracy was better than the performance of SCr (AUC=0.73) and quite similar to that of CysC (AUC=0.91), which is considered a marker with a great sensitivity for detecting impaired renal function and kidney tubular injury.

- The prognostic value of early values of MDA on long-term allograft function (one-year after kidney transplantation) as evaluated by SCr was tested by multivariable regression analysis. The MDA levels measured on day-7 were independent predictors of the one-year graft function after controlling for established variables that usually affect graft function. The levels of MDA before Kidney transplantation and on post-transplant remaining days were not significant predictors of the one-year SCr.

ERRATA:

Page 75. Paper III, Fig. 1, after the ROC curves graph and before the legend, the table with the biomarkers AUCs was not printed in the paper.

	AUC (95% CI)	***P*-Value**
Day 1 (8-to-12 h after surgery)		
MDA (µmol/L)	0.90 (0.81 - 0.99)	< 0.001
Serum Creatinine (mg/dl)	0.73 (0.58 - 0.89)	0.012
Serum Cystatin C (mg/L)	0.91 (0.82 - 1.00)	< 0.001
Change from pre-transplant to first day after KTx		
MDA (µmol/L)	0.84 (0.70 - 0.97)	< 0.001
Serum Creatinine (mg/dl)	0.69 (0.52 - 0.87)	0.036

CLINICAL AND TRANSLATIONAL RESEARCH

Oxidative Stress in Kidney Transplantation: Malondialdehyde Is an Early Predictive Marker of Graft Dysfunction

Isabel Fonseca,[1,2,3,6] *Henrique Reguengo,*[4] *Manuela Almeida,*[1,2] *Leonídio Dias,*[1] *La Salete Martins,*[1,2] *Sofia Pedroso,*[1] *Josefina Santos,*[1,2] *Luísa Lobato,*[1,2] *António Castro Henriques,*[1,2] *and Denisa Mendonça*[3,5]

Background. Oxidative stress is one of the most important components of the ischemia-reperfusion process after kidney transplantation (KTx) and increases with graft dysfunction.

Methods. This prospective study was conducted on 40 consecutive KTx recipients to evaluate time-dependent changes in oxidative stress-related parameters within the first week after KTx and to assess their performance in predicting delayed graft function (DGF=dialysis requirement during initial posttransplant week) and graft function at 1 year. Blood samples were collected before (day 0) and after KTx (days 1, 2, 4, and 7). Total antioxidant capacity, plasma levels of malondialdehyde (MDA), and activities of glutathione peroxidase, glutathione reductase and superoxide dismutase were measured. Multivariable linear mixed and linear regression models, receiver-operating characteristic (ROC), and areas under ROC curves (AUC-ROC) were used.

Results. At all time points after KTx, mean MDA levels were significantly higher in patients developing DGF (n=18). Shortly after KTx (8–12 hr), MDA values were higher in DGF recipients (on average, +0.16 μmol/L) and increased further on following day, contrasting with prompt functioning recipients. Day 1 MDA levels accurately predicted DGF (AUC-ROC=0.90), with a performance higher than SCr (AUC-ROC=0.73) and similar to cystatin C (AUC-ROC=0.91). Multivariable analysis revealed that MDA levels on day 7 represented an independent predictor of 1-year graft function. Antioxidant enzyme activities were not significantly changed during the study period and were not predictors of 1-year graft function.

Conclusions. Increased MDA levels on day 1 after KTx might be an early prognostic indicator of DGF, and levels on day 7 might represent a useful predictor of 1-year graft function.

Keywords: Oxidative stress, Malondialdehyde, Kidney transplantation, Kidney graft dysfunction.

(*Transplantation* 2014;97: 1058–1065)

Ischemia-reperfusion (I/R) injury is a complex phenomenon in kidney transplantation (KTx) that can cause graft dysfunction and determine both the early and long-term outcomes of transplant recipients. Oxidative stress is one of the most important components of I/R process (*1–3*). Reactive oxygen species (ROS) are products of normal cellular metabolism that are completely inactivated by antioxidant defense mechanisms during physiological conditions.

This study was funded by Astellas Farma Portugal. Astellas Farma Portugal contributed with financial funding sources for reagents acquisition and had no role in the design or conduct of the study, collection, management, analysis, interpretation of the data or in the preparation, review, or approval of the manuscript.

The authors declare no conflicts of interest.

[1] Nephrology and Kidney Transplantation Department, Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal.

[2] Unit for Multidisciplinary Investigation in Biomedicine (UMIB), Porto, Portugal.

[3] Institute of Public Health (ISPUP), University of Porto, Porto, Portugal.

[4] Chemical Chemistry Department, Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal.

[5] Department of Population Studies, Institute of Biomedical Sciences Abel Salazar (ICBAS) University of Porto, Porto, Portugal.

[6] Address for Correspondence: Isabel Fonseca, Nephrology and Kidney Transplantation Department, Centro Hospitalar do Porto, Hospital de Santo António , Largo Prof. Abel Salazar 4099-001 Porto, Portugal.

E-mail: isabelf27@gmail.com

Department and Institution to which the work should be attributed: Nephrology and Kidney Transplantation Department, Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal

I.F. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: I.F. Acquisition of data and patient recruitment: I.F., M.A., L.D., S.P., J.S., A.C.H. Technical support and laboratorial analysis: H.R., I.F. Statistical analysis: I.F., D.M. Analysis and interpretation of data: I.F., D.M. Drafting of the manuscript: I.F. Critical revision of the manuscript for important intellectual content: D.M., H.R., A.C.H., L.L. All authors approved the final version. Obtained funding: A.C.H. Study supervision: A.C.H., D.M., L.L.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

Received 27 August, 2013. Revision requested 14 September 2013.
Accepted 4 November 2013.

ISSN: 0041-1337/14/9710-1058
DOI: 10.1097/01.TP.0000438626.91095.50

The antioxidant defense system can be predominantly divided into endogenous enzymes, such as superoxide dismutases (SOD), catalases, glutathione reductases (GR) and peroxidases (GPx), and exogenous small molecules, such as carotenoids and vitamins A, C, and E (*4*). In some pathologic conditions, an imbalance between ROS generation and antioxidant capacity can result in enhanced ROS activity and oxidative stress (*5*).

Markers of oxidative stress, including elevated levels of malondialdehyde (MDA) and reduced antioxidant activity,

TABLE 1. Summary of baseline and clinical characteristics in kidney transplant donors and recipients (total sample and categorized by delayed or prompt graft function)

	Total (n=40)	DGF (n=18)	Non-DGF (n=22)	*P*
Donor				
Age (yr)	51.2±11.4	51.1±13.4	51.2±9.9	0.172
Male sex	26 (65)	14 (78)	12 (54.5)	0.125
Living donor	11 (27.5)	3 (16.7)	8 (36.4)	0.165
Expanded criteria donors	3 (7.5)	1 (5.6)	2 (9.1)	0.541
Serum creatinine (mg/dL)	0.81±0.18	0.85±0.21	0.78±0.16	0.318
Donor-recipient				
HLA mismatches	3.39±1.24	3.38±1.07	3.41±1.46	0.941
Cold ischemia time (hr)	12.1±7.9	15.2±7.8	9.6±7.3	0.035*
Living donor	2.8±0.5	2.5±0.5	3.0±0.5	0.204
Deceased donor	16.2±5.9	18.1±5.1	14.1±6.2	0.088
Recipient				
Age (yr)	49.2±15.2	56.3±10.9	43.3±15.9	0.006*
Male sex	26 (65)	11 (61)	15 (68)	0.641
White	40 (100)	18 (100)	22 (100)	—
BMI (kg/m^2)	24.8±4.9	26.2±4.4	23.6±5.0	0.091
Previous transplant	2 (5)	0 (0)	2 (9.1)	
Time on dialysis (yr)	4.4±4.7	5.6±6.2	3.4±2.3	0.135
Pretransplant therapy				
Dialysis	38 (95)	18 (100)	20 (90.9)	0.296
Preemptive transplantation	2 (5)	0 (0)	2 (9.1)	
Cause of kidney disease				
IgA nephropathy	7 (17.5)	2 (11.1)	5 (22.7)	—
Glomerulonephritis	6 (15.0)	4 (22.2)	2 (9.1)	—
Diabetic nephropathy	5 (12.5)	3 (16.7)	2 (9.1)	—
Autosomal dominant polycystic kidney disease	3 (7.5)	3 (16.7)	0 (0)	—
Unknown	4 (10.0)	1 (5.6)	3 (13.6)	—
Others	15 (37.5)	5 (27.8)	10 (45.5)	—
Peak PRA (%)	5.5 ± 15.1	5.0 ± 15.0	5.9 ± 15.5	0.853
0	29 (72.5)	14 (77.8)	15 (68.2)	—
1–25	8 (20.0)	3 (16.7)	5 (22.7)	—
26–75	3 (7.5)	1 (5.6)	2 (9.0)	—
Current PRA (%)	2.3 ± 8.6	3.1 ± 11.7	1.6 ± 4.9	0.585
0	34 (85)	15 (83.3)	19 (86.4)	—
1–25	5 (12.5)	2 (11.1)	3 (13.6)	—
26–50	1 (2.5)	1 (5.6)	0 (0)	—
Induction regimen				
Antithymocyte globulin (ATG-F)	4 (10)	1 (5.6)	3 (13.6)	0.613
Basiliximab/Daclizumab	30 (75)	14 (77.8)	16 (72.7)	0.789
Immunosuppression at time of discharge				
Steroids	38 (95.0)	18 (100)	20 (90.9)	0.296
Tacrolimus	38 (95.0)	17 (94.4)	21 (95.5)	0.886
Cyclosporine A	2 (0.05)	1 (5.6)	1 (5.6)	0.884

Values are expressed as mean±standard deviation or absolute numbers and percentages. Comparisons between groups of continuous variables were done using parametric (*t* test) or nonparametric (Mann-Whitney) tests; associations between categorical variables were analyzed using the χ^2 test and Fisher's exact test as appropriate; *$P<0.05$.

HLA, human leukocyte antigen; BMI, body mass index; PRA, panel reactive antibody.

have been reported in renal patients (*6–9*). The restoration of kidney function after KTx can improve oxidative stress (*10*), but certain studies (*11, 12*) have reported increased systemic biomarkers of oxidative stress in KTx recipients, particularly in the early phase (*13, 14*) and thereafter, coinciding with chronic allograft dysfunction (*11, 15–18*). Despite a significant amount of literature on oxidative stress and renal disease, data regarding KTx in the early stages remain limited. Therefore, we investigated the time-dependent changes in the antioxidant defense system during the first week after transplantation by measuring the overall antioxidant status (TAS) and the activity of the predominant antioxidant enzymes as a response to lipid peroxidation evaluated by MDA levels.

The purposes of this study were as follows:

to assess whether oxidative markers differ between patients (pretransplant and 1 week posttransplant) and control subjects (healthy blood donors);
to evaluate longitudinal changes of MDA, TAS, SOD, GPx, and GR within the first week after KTx and identify factors associated with these changes;
to investigate the association of MDA/antioxidant parameters with DGF (defined as dialysis requirement within the first posttransplant week) and their accuracy in predicting DGF; and
to examine the relationship between any of the oxidative markers measured during the first week posttransplant and the 1-year allograft function, evaluated by serum creatinine (SCr) levels.

RESULTS

Study Cohort

During recruitment, 42 patients were consecutively enrolled. Two recipients had primary graft failure and were excluded during the first 2 days. Therefore, the final study sample included 40 patients. Baseline demographical and transplant data are shown in Table 1.

Oxidative Stress Markers

We initially compared oxidative markers evaluated in the 40 ESRD patients scheduled for KTx with those of 30 healthy subjects with similar ages (a control group of blood donors). Before KTx, the patients presented with significantly increased mean (SD) MDA levels (0.40 [0.12] vs. 0.26 [0.09] μmol/L, $P<0.01$), TAS (1.79 [0.19] vs. 1.39 [0.53], $P<0.001$), SOD (1971 [630] vs. 1208 [254] U/g Hb, $P<0.001$) and GR (63 [12] vs. 52 [7.0] U/L, $P<0.001$) compared with controls. No significant differences were detected in GPx.

The evolution of oxidative parameters during the first posttransplant week is summarized in Table 2. Compared with before transplant, mean (SD) MDA levels significantly decreased at first day (0.40 [0.12] vs. 0.36 [0.12] μmol/L, $P=0.031$), and a reduction of approximately 28% was observed on the seventh posttransplant day (0.40 [0.13] to 0.28 [0.13] μmol/L, $P<0.001$). Levels of TAS, SOD, GPx, and GR did not exhibit any significant changes within the first posttransplant week.

None of the oxidative stress markers differed significantly between male and female patients at any time point. Mean MDA levels were increased in deceased donor recipients at all time points, although the increases were only statistically significant on second and fourth days. No significant differences were found in antioxidant parameters.

Recipient age was positively correlated with MDA levels at days 4 and 7 (respectively, $r=0.46$, $P=0.004$; and $r=0.39$, $P=0.013$). Time on dialysis, donor age, and cold ischemia time were not correlated with MDA levels or with any antioxidant marker. Levels of MDA and SCr, but not of the antioxidant markers, were positively correlated at most of time points (data not shown).

TABLE 2. Time-course of oxidative stress biomarkers within the first week after kidney transplantation

		Prior-KTx	1st day*	2nd day	4th day	7th day
		Mean (SD)	(n=40)	(n=40)	(n=40)	(n=40)
MDA (μmol/L)	Overall	0.40 (0.12)	0.36 (0.12)	0.28 (0.10)	0.29 (0.13)	0.28 (0.13)
	DGF (n=18)	0.42 (0.12)	0.45 (0.10)	0.33 (0.10)	0.40 (0.13)	0.37 (0.13)
	Non-DGF (n=22)	0.39 (0.12)	0.29 (0.09)	0.24 (0.08)	0.23 (0.07)	0.19 (0.05)
TAS (mmol/L)	Overall	1.79 (0.19)	1.74 (0.21)	1.68 (0.35)	1.73 (0.37)	1.77 (0.30)
	DGF (n=18)	1.80 (0.16)	1.73 (0.24)	1.70 (0.25)	1.82 (0.21)	1.89 (0.24)
	Non-DGF (n=22)	1.78 (021)	1.76 (0.19)	1.74 (0.22)	1.75 (0.28)	1.66 (0.32)
SOD (U/g Hb)	Overall	1966 (638)	1894 (596)	1984 (578)	1947 (457)	1997 (580)
	DGF (n=18)	1842 (559)	1837 (622)	1876 (538)	1928 (439)	2158 (604)
	Non-DGF (n=22)	2068 (691)	1943 (584)	2071 (608)	1959 (478)	1944 (413)
GR (U/L)	Overall	63 (12)	50 (14)	51 (17)	56 (16)	62 (14)
	DGF (n=18)	66 (12)	54 (19)	57 (20)	63 (18)	69 (15)
	Non-DGF (n=22)	61 (12)	47 (9)	48 (7)	53 (10)	56 (10)
GPx (U/g Hb)	Overall	58 (15)	59 (13)	62 (15)	62 (14)	60 (14)
	DGF (n=18)	58 (13)	59 (11)	62 (14)	64 (15)	60 (14)
	Non-DGF (n=22)	58 (17)	60 (15)	62 (17)	60 (14)	60 (14)

*1st day=8 to 12 hr after surgery; the values are the mean and standard deviation.

KTx, kidney transplantation; MDA, malondialdehyde; TAS, total antioxidant status; SOD, superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; SD, standard deviation.

Delayed Graft Function and Acute Rejection

Eighteen (45%) and 22 (55%) patients had DGF and prompt graft function, respectively. The DGF rate was higher in grafts from deceased donors, but this difference was not statistically significant (51.7% vs. 27.3%, *P*=0.286). In terms of traditional DGF predictors and except for cold ischemia time, no significant differences were found between DGF/non-DGF in relation to baseline characteristics and induction therapy (Table 1). The mean age was significantly higher in patients with DGF (56 [11] vs. 43 [16] yr, *P*=0.006).

Ten recipients had an acute rejection episode during inpatient hospitalization for transplantation, and acute rejection was more frequently diagnosed in patients with DGF than in those with prompt function (44% vs. 9%, *P*=0.025).

DGF and Longitudinal Changes in Oxidative Stress Markers

Before transplantation, no significant differences were found between patients with DGF or non-DGF regarding any of the evaluated oxidative stress markers. After transplantation, mean MDA levels were consistently higher in DGF patients at all time points, compared with non-DGF recipients (Table 2). No differences were found between DGF and non-DGF recipients in relation to antioxidant parameters.

Longitudinal Changes in MDA Levels According to Graft Function

A linear mixed-effects model was used to analyze the longitudinal changes in MDA of the 2 groups of patients (DGF/non-DGF), by controlling for variables found to be associated with MDA by bivariate analysis (donor status and recipient age) and confirmed the independent association of DGF with and MDA levels. Donor status and recipient age lost their statistical significance and were removed from the final model. Time measurements of MDA and DGF were the only independent factors associated with MDA levels (Table 3). Delayed graft function was significantly associated with MDA levels: recipients with prompt function presented reduced average MDA levels at all time points. According to our estimation, the first MDA values after transplantation were 0.16 μmol/L higher in DGF patients. A significant interaction between time of measurement and DGF confirmed that the pattern of longitudinal changes in MDA levels depend on whether the recipient had DGF.

Because DGF occurs more frequently in KTx from deceased donors, we performed the same analysis considering only deceased donor transplants, and the results were similar (see **SDC**, http://links.lww.com/TP/A919). According to our estimation and after excluding living donors, the first MDA levels after KTx were, on average, 0.144 μmol/L higher in DGF patients who underwent a deceased-donor transplant.

The effect of DGF on the progression of antioxidant parameters over time was not statistically significant, even when we considered only deceased-donors transplants.

Prognosis of DGF by Oxidative Stress Markers (ROC Analysis)

Receiver-operating characteristic (ROC) analyses were performed to assess the potential of oxidative markers to predict DGF. Only MDA levels predicted the need for dialysis within the first week. The MDA levels on day 1 represented an optimal predictor for the early diagnosis of DGF (AUC=0.90), as well as the changes in MDA levels between preoperative and first posttransplant day (AUC=0.84) (Fig. 1). The diagnostic performance of MDA on day 1 was better than diagnostic performance of SCr (AUC=0.73) and similar to that of cystatin C (CystC, AUC=0.91), which is considered a marker with greater sensitivity for the detection of impaired renal function. The reduction ratio in MDA levels between pretransplant and day 1 resulted in an AUC of 0.84 for identifying DGF, which was better than the reduction ratio of SCr on the same day (AUC=0.69). In analyzing

TABLE 3. Results of the final linear mixed model for dependent variable MDA levels (n = 194 observations derived from 40 patients)

	Estimate	*P*	95% CI	
Intercept	0.368	<0.001	0.318	0.418
Graft function				
DGF=0 (immediate graft function)	–0.170	<0.001	–0.238	–0.102
DGF=1 (with DGF - reference)	0	—	—	—
Time				
Time 0 (pretransplant)	0.053	0.089	–0.008	0.115
Time 1 (1st day)	0.079	0.012	0.018	0.141
Time 2 (2nd day)	–0.034	0.279	–0.095	0.028
Time 3 (4th day)	0.030	0.363	–0.036	0.097
Time 4 (7th day- reference)	0	—	—	—
Time*DGF				
Time 0*DGF=0	0.134	0.002	0.050	0.217
Time 1*DGF=0	0.013	0.257	–0.070	0.097
Time 2*DGF=0	0.077	0.295	–0.007	0.161
Time 3*DGF=0	0.029	0.039	–0.058	0.116
Time 4*DGF=0 (reference)	0	—	—	—

MDA, Malondialdehyde; DGF, delayed graft function.

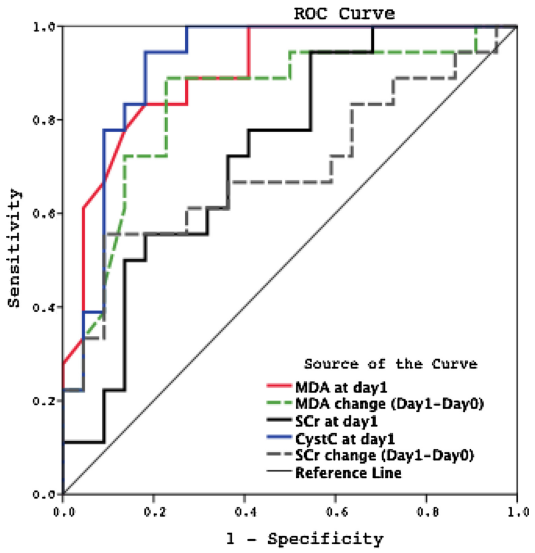


FIGURE 1. Receiver-operating characteristic curves for plasma MDA, serum creatinine, and cystatin C levels measured at the first day after KTx and changes in MDA and serum creatinine levels from pretransplant to day-1 after KTx for predicting delayed graft function. The table lists the areas under the ROC curves of MDA, serum creatinine, and serum cystatin C for predicting DGF, as well as the AUCROC of MDA and serum creatinine changes between baseline and first day after transplant. MDA, malondialdehyde; SCr, serum creatinine; Cyst, serum cystatin C; MDA or SCr change (day 1–day 0), Serum creatinine or MDA reduction rate between pretransplant and the first day after transplant (the difference between MDA or SCr on day 1 and day 0, divided by MDA or SCr on day 0, multiplied by 100); AUC, area under the ROC curves.

the ROC curve of MDA on day 1, the optimal sensitivity and specificity occurred at a value of 0.365 μmol/L (sensitivity, 83%; specificity, 82%; positive and negative predictive value, 82 and 71, respectively).

During the First Year After KTx

Within the first year after KTx, 10 KTx recipients were rehospitalized, accounting for a total of 19 hospital admissions. The causes of rehospitalization were infection in five admissions (predominantly urinary tract infections), renal dysfunction in six, and nonrenal causes in the remaining eight admissions (suicidal ideation, acute pulmonary edema, and neutropenia). Records from the acute rejection episodes throughout the first posttransplant year were reviewed, and only one patient was rehospitalized at 1 month after KTx with an acute rejection episode. At 1 year, all of the patients were alive, but two grafts of DGF recipients were lost.

Predictive Value of MDA Levels on 1-Year Allograft Function

At 1 year after KTx, the median (IQR) SCr was significantly higher in patients with DGF (1.58 [1.20–2.52] vs. 1.26 [1.05–1.52] mg/dL, P=0.049) and a correlation with MDA at day 7 was found (Fig. 2). The prognostic value of early MDA values on long-term allograft function (1 year after KTx) was tested using multivariable analysis, including all patients (DGF and non-DGF). In multivariable linear regression models for 1-year SCr, MDA levels measured on day 7 were independent predictors of 1-year graft function after controlling for established variables that generally affect graft function, including acute rejection and rehospitalizations occurring during the first posttransplant year (Table 4). Levels of MDA before KTx and on remaining posttransplant days were not significant predictors of 1-year SCr.

DISCUSSION

In this prospective cohort study, we report the independent association of high levels of plasma MDA with DGF with poor 1-year allograft function. To the best of our knowledge, this study is the first to demonstrate this association in KTx recipients.

Oxidative stress is involved in the pathophysiology of renal injury in I/R (*1, 2, 19*). As in other clinical conditions, if the kidney scavenging capacity is insufficient for an excess of ROS production, such an oxidative imbalance might trigger an inflammatory response within the transplanted organ, leading to tissue damage and graft dysfunction (*2, 13, 20*). Because of the composition of renal lipids, which predominantly comprise long-chain, polyunsaturated fatty acids, lipid peroxidation represents one of the most widespread hypothesized causes of ROS-mediated cell injury (*21*). Despite the controversy of whether lipid peroxidation is the cause or an epiphenomenon of injury, the fact is that increased lipid peroxidation is observed in I/R injury. Moreover, MDA is the principal product of polyunsaturated fatty acid peroxidation, reflecting the I/R stress of grafts (*5, 22*). In our study, recipients who developed DGF presented increased MDA levels during the first week after KTx, which seem to reflect the postischemic tissue damage of DGF kidneys. Compared with pretransplant, these patients presented higher MDA levels at 8 to 12 hr after KTx, in contrast to recipients with prompt graft function whose MDA levels continuously decreased throughout the

TABLE 4. Significant predictors of serum creatinine at 1 year after kidney transplantation

	Regression coefficient	P	95% CI
	Serum creatinine at 1-year posttransplantation (ln)		
Time on dialysis (ng/mL)	0.048	<0.001	0.024–0.072
MDA measured on day-7 (μmol/L)	1.338	0.003	0.475–2.201
Rehospitalizations (yes vs. no)	0.361	0.007	0.107–0.615

Results are given by multiple linear regression (a stepwise method) after including donor status, recipient and donor age, pretransplant time on dialysis, rehospitalizations and acute rejection episodes throughout the first year; serum creatinine (ln) at 1-year after transplant as the dependent variable.
MDA, malondialdehyde.

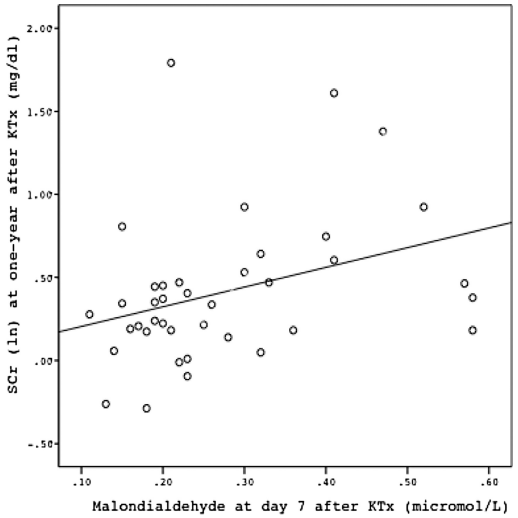


FIGURE 2. Relationship between serum creatinine and plasma malondialdehyde at day 7 after kidney transplantation (r=0.346, P=0.031). KTx, kidney transplantation; SCr, serum creatinine.

week. The independent association of DGF with longitudinal changes in MDA was confirmed using a general linear mixed model approach, which also corroborated that DGF can model the trajectory of MDA changes after KTx.

Our results not only establish MDA as an early marker of DGF but also demonstrate its predictive value as early as 8 to 12 hr after KTx in terms of the evolution of graft function and the need for dialysis during the first week. In regard to clinical application, a new biomarker should be more accurate in predicting DGF than the current SCr. After this, ROC analysis showed that MDA levels are better suited than SCr for predicting the need of renal replacement therapy within the first week after KTx. Similar to serum CystC, MDA levels on day 1 were highly accurate in predicting DGF and performed better than SCr. This emphasizes the clinical value of MDA levels as a diagnostic marker for the prediction of DGF, facilitating an earlier diagnosis compared with SCr.

Only a highly effective antioxidant system can counteract the deleterious hydroxyl radicals formed during lipid peroxidation. A wide range of protective substances, such as antioxidant enzymes, might potentially elicit a protective effect by limiting the production of ROS and the damage of oxidative stress after I/R injury of a kidney graft. Conflicting results have reported on the activities of antioxidant enzymes in KTx patients. Levels of antioxidant enzymes have been reported to increase (*23, 24*), decrease (*12, 16*), or remain unchanged (*25, 26*) after KTx. Because of this lack of consensus, we aimed to examine the changes of antioxidant activity during the early phase of KTx. Compared with healthy controls, our patients presented with significantly increased SOD and GR levels before KTx, likely in response to significant oxidative stress levels in ESRD patients. However, no significant changes were found after KTx, even when stratifying by graft function.

The evaluation of TAS has been used as a biological marker for monitoring oxidative stress. Measuring TAS allows for the detection of the overall antioxidant capacity, including the contribution of as of yet unknown antioxidants and the synergism between them (*27*). In our study, TAS levels did not exhibit any significant changes during the first posttransplant week, even when we stratified the patients according to DGF. Although oxidative stress expressed by MDA levels is most significantly pronounced in DGF patients, our study highlights the observation that during the first posttransplant week, the overall antioxidant status and potential protection exerted by the antioxidant enzymes are not enhanced to counteract the intensified oxidative stress, specifically in DGF patients.

Immunosuppressive therapy, particularly cyclosporine, represents an additional potential source of ROS generation and enhanced renal lipid peroxidation after KTx (*15, 28*). In our study, the effect of immunosuppression on plasma MDA and antioxidant parameters was not assessed, as only one patient was on a combined therapy with cyclosporine.

In various studies, it has been reported that oxidative stress occurring in KTx might be implicated in the pathophysiology of chronic transplant dysfunction (*1, 11, 15, 17, 29*). Djamali et al. (*30*) suggested that ROS represents an important fibrogenic factor in chronic allograft nephropathy because oxidative stress is increased in the presence of the interstitial fibrosis and tubular atrophy that generally precedes chronic allograft failure. In experimental models of chronic allograft tubular atrophy/interstitial fibrosis, increased intragraft MDA levels were detected, reflecting lipid peroxidation (*31*). Therefore, we verified the effects of MDA levels evaluated within the first week on 1-year posttransplant allograft function. Together with time on dialysis and rehospitalizations during the first year, MDA levels at day 7 were the best predictors of 1-year SCr. Higher MDA levels on day 7 were associated with worse graft function at 1 year, suggesting that oxidative damage reflected by increased MDA levels on day 7 will reflect long-term injury.

This study has several strengths. We used a prospective and longitudinal study design to determine the effects of DGF on the progression of oxidative markers over time. Most of the previous oxidative stress studies on KTx were cross-sectional and included only stable patients. Longitudinal studies are more helpful in understanding how subtle associations between factors of interest change over time, and we used this methodology to consider five measurements of each oxidative stress marker. Uncertainty remains concerning the determination of oxidative stress in KTx and the interpretation of the potential variability. However, our study highlights the importance of studying oxidative stress according to graft function because DGF can significantly modify the trajectory of MDA changes. To the best of our knowledge, this is the first study to demonstrate that MDA levels are strongly associated with DGF and with poorer 1-year graft function.

Regardless of its several mentioned strengths, this study has some limitations. This is a single centre study with a relatively small sample size. Despite the encouraging results found, the accuracy of MDA levels as a diagnostic marker of renal graft injury and prognostic value of MDA for DGF after KTx needs to be assessed in a larger cohort and in other centers and transplant recipients.

In KTx, numerous diagnostic biomarkers have been evaluated in the past decade, but, so far, evidence to support

their use in routine practice is limited. The discovery of novel biomarkers can be complex and costly. In this study, we demonstrated that a novel marker predicted who would develop DGF with about the same degree of accuracy of serum CystC and both with a diagnostic performance superior to serum creatinine. Undoubtedly, CystC displays several good characteristics that make it a viable biomarker for early detection of DGF. Nonetheless, and particularly during the first week when high doses of corticosteroids are used, glucocorticoid medication can be shortcoming in using serum CystC in KTx, and it is important to take this into account when interpreting this serum marker. Thus, a combination of biomarkers may be more valuable for the diagnosis of DGF and prognosis of graft function. Because DGF is a critical early insult to the renal allograft that augments the risk of long-term graft loss, and it is a complex process with multiple underlying pathogenic mechanisms and confounding risk factors, it can be prudent to predict DGF with more than a single biomarker, at least in some situations. MDA can be a valuable marker as an alternative or as a complement in the risk prediction, not only in relation to serum CystC and any other serum/plasma markers but also regarding urine biomarkers, like neutrophil gelatinase-associated lipocalin, that cannot be measured if a urine sample cannot be taken, particularly during transient anuria that commonly occurs after KTx.

In conclusion, intensified oxidative stress persists during the early phase of transplant, particularly in DGF recipients. The antioxidant enzymes did not counterbalance the overload of ROS by a compensatory increase in their activities. The present study showed that MDA is a novel and a reliable biomarker for the prediction of early and long-term graft damage.

SUBJECTS AND METHODS

Study Design and Patient Population

Consecutive patients with end-stage renal disease (ESRD), undergoing living or deceased KTx at the Nephrology and Kidney Transplantation Department of the Centro Hospitalar do Porto between December 2010 and May 2011 were prospectively enrolled. Patients younger than 18 years or who required multiorgan transplants were not included. After transplant, recipients with primary graft failure related to surgical causes were excluded. The institutional review board of Centro Hospitalar do Porto approved the study. Each participant provided informed consent before enrollment.

Data Collection

At time of transplantation, several demographical and clinical parameters were collected. During the first posttransplant year, the rehospitalizations of KTx recipients were registered, as well as the length of the hospital stays and outcomes (functioning allograft or graft failure).

Sampling and Laboratory

Blood samples for determining oxidative stress parameters were collected as follows: 3 to 6 hr before transplant surgery (pretransplant); on the following morning, approximately 8 to 12 hr after graft reperfusion (day 1); and then at second (day 2), fourth (day 4), and seventh day (day 7) after transplant, for a total of five samples per patient. Blood samples were taken by conventional procedures and immediately centrifuged. All samples were aliquoted and frozen within 1 hr after collection and stored at –80ôºC until further assay.

Measurements of SCr were performed by Jaffé method (Roche Diagnostics), and CystC was measured with a particle enhanced immunonephelometric method (Siemens Diagnostics) at the same time points as oxidative markers.

Plasma levels of MDA were measured using a commercial high-performance liquid chromatography kit (Chromsystems). superoxide dismutase levels were measured in erythrocytes according to a protocol previously described by Beauchamp and Fridovich (*32*) using the RANSOD kit; GR, GPx, and TAS levels were measured in plasma/serum using a Randox Laboratories kit.

Definition of Variables

Delayed graft function was defined by the need for dialysis during the first week. "Prompt" function (non-DGF) was considered if no dialysis was required during the first posttransplantation week.

Graft function at 1 year was evaluated by the average of the two SCr levels measured at 1 year posttransplant. Two grafts were lost at the seventh and eighth months, and the last SCr presented by these patients before the restart of dialysis was considered as being the 1-year SCr.

Statistics

Distributions of continuous variables were analyzed, and Kolmogorov-Smirnov tests were performed to assess their deviation from Normal distribution. Quantitative variables were summarized as the mean and standard deviation (SD), or as median and 25th and 75th quartiles (interquartile range [IQR]) for variables exhibiting skewed distributions. Categorical variables were reported as percentages.

Statistical analysis was performed in four steps. First, a cross-sectional bivariate analysis was performed to compare groups and to study the association between oxidative stress markers and demographic/clinical variables (*t* test). Correlations were assessed using Pearson correlation.

Second, a linear mixed-effects model was used to study the association of DGF with serial changes of each oxidative marker, controlling for variables associated by bivariate analysis. The interaction between DGF and the time-course measurement of oxidative markers were included in the model, as such a significant interaction would suggest that DGF affects the levels and trajectory of each marker.

Third, ROC analysis was performed to estimate the sensitivity and specificity of MDA levels (as well as SCr and CystC) to predict DGF. The optimal cutoff points were determined by maximizing the sum of sensitivity and specificity.

Fourth, multivariable stepwise linear regression was performed to assess the independent association of MDA levels with SCr at 1 year posttransplantation, including variables that generally predict graft function (donor status, recipient and donor age, pretransplant time on dialysis, rehospitalization, and acute rejection episodes throughout the first year). Linear regression models used log-transformed 1-year SCr levels as the dependent variable. To avoid collinearity, each time point of MDA was included separately in the different models.

Statistical analyses were performed using SPSS version 21.0, and a significance level of 0.05 was considered significant.

ACKNOWLEDGMENTS

The authors thank Astellas Pharma Portugal for their valuable contribution in supporting the acquisition of reagents for measuring malondialdehyde and all of the antioxidant parameters.

The authors also thank Dr. Maria Luís Cardoso for the technical assistance in laboratorial analysis and to the nursing staff for the commitment and crucial help in sample collection. The authors also thank Drs. António Cabrita and José Carlos Oliveira for allowing the study in their departments (the Nephrology and Kidney Transplantation and Chemical Chemistry Departments, respectively, of the Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal).

REFERENCES

1. Kim J, Seok YM, Jung KJ, et al. Reactive oxygen species/oxidative stress contributes to progression of kidney fibrosis following transient ischemic injury in mice. *Am J Physiol Renal Physiol* 2009; 297: F461.

2. Hariharan N, Zhai P, Sadoshima J. Oxidative stress stimulates autophagic flux during ischemia/reperfusion. *Antioxid Redox Signal* 2011; 14: 2179.
3. Dolegowska B, Blogowski W, Domanski L. Association between the perioperative antioxidative ability of platelets and early post-transplant function of kidney allografts: a pilot study. *PLoS One* 2012; 7: e29779.
4. Valko M, Izakovic M, Mazur M, et al. Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem* 2004; 266: 37.
5. Valko M, Rhodes CJ, Moncol J, et al. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 2006; 160: 1.
6. Oberg BP, McMenamin E, Lucas FL, et al. Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* 2004; 65: 1009.
7. Miguel A, Linares M, Perez A, et al. Evidence of an increased susceptibility to lipid peroxidation in red blood cells of chronic renal failure patients. *Nephron* 1988; 50: 64.
8. Mimic-Oka J, Simic T, Djukanovic L, et al. Alteration in plasma antioxidant capacity in various degrees of chronic renal failure. *Clin Nephrol* 1999; 51: 233.
9. Paul JL, Sall ND, Soni T, et al. Lipid peroxidation abnormalities in hemodialyzed patients. *Nephron* 1993; 64: 106.
10. Simmons EM, Langone A, Sezer MT, et al. Effect of renal transplantation on biomarkers of inflammation and oxidative stress in end-stage renal disease patients. *Transplantation* 2005; 79: 914.
11. Simic-Ogrizovic S, Simic T, Reljic Z, et al. Markers of oxidative stress after renal transplantation. *Transpl Int* 1998; 11(Suppl 1): S125.
12. Campise M, Bamonti F, Novembrino C, et al. Oxidative stress in kidney transplant patients. *Transplantation* 2003; 76: 1474.
13. Ardalan MR, Estakhri R, Hajipour B, et al. Erythropoietin ameliorates oxidative stress and tissue injury following renal ischemia/reperfusion in rat kidney and lung. *Med Princ Pract* 2013; 22: 70.
14. Zahmatkesh M, Kadkhodaee M, Mahdavi-Mazdeh M, et al. Oxidative stress status in renal transplant recipients. *Exp Clin Transplant* 2010; 8: 38.
15. Djamali A. Oxidative stress as a common pathway to chronic tubulointerstitial injury in kidney allografts. *Am J Physiol Renal Physiol* 2007; 293: F445.
16. Cristol JP, Vela C, Maggi MF, et al. Oxidative stress and lipid abnormalities in renal transplant recipients with or without chronic rejection. *Transplantation* 1998; 65: 1322.
17. Raj DS, Lim G, Levi M, et al. Advanced glycation end products and oxidative stress are increased in chronic allograft nephropathy. *Am J Kidney Dis* 2004; 43: 154.
18. Vos IH, Joles JA, Rabelink TJ. The role of nitric oxide in renal transplantation. *Semin Nephrol* 2004; 24: 379.
19. Haugen E, Nath KA. The involvement of oxidative stress in the progression of renal injury. *Blood Purif* 1999; 17: 58.
20. Lee DM, Jackson KW, Knowlton N, et al. Oxidative stress and inflammation in renal patients and healthy subjects. *PLoS One* 2011; 6: e22360.
21. Ozbek E. Induction of oxidative stress in kidney. *Int J Nephrol* 2012; 2012: 465897.
22. Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metab Cardiovasc Dis* 2005; 15: 316.
23. Whitin JC, Tham DM, Bhamre S, et al. Plasma glutathione peroxidase and its relationship to renal proximal tubule function. *Mol Genet Metab* 1998; 65: 238.
24. Zachara BA, Wlodarczyk Z, Masztalerz M, et al. Selenium concentrations and glutathione peroxidase activities in blood of patients before and after allogenic kidney transplantation. *Biol Trace Elem Res* 2004; 97: 1.
25. Vostalova J, Galandakova A, Svobodova AR, et al. Time-course evaluation of oxidative stress-related biomarkers after renal transplantation. *Ren Fail* 2012; 34: 413.
26. Domanski L, Dolegowska B, Safranow K, et al. Activity of CuZn-superoxide dismutase, catalase and glutathione peroxidase in erythrocytes in kidney allografts during reperfusion in patients with and without delayed graft function. *Clin Transplant* 2006; 20: 67.
27. Abuja PM, Albertini R. Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins. *Clin Chim Acta* 2001; 306: 1.
28. Perrea DN, Moulakakis KG, Poulakou MV, et al. Correlation between oxidative stress and immunosuppressive therapy in renal transplant recipients with an uneventful postoperative course and stable renal function. *Int Urol Nephrol* 2006; 38: 343.
29. Albrecht EW, Stegeman CA, Tiebosch AT, et al. Expression of inducible and endothelial nitric oxide synthases, formation of peroxynitrite and reactive oxygen species in human chronic renal transplant failure. *Am J Transplant* 2002; 2: 448.
30. Djamali A, Reese S, Yracheta J, et al. Epithelial-to-mesenchymal transition and oxidative stress in chronic allograft nephropathy. *Am J Transplant* 2005; 5: 500.
31. Gottmann U, Oltersdorf J, Schaub M, et al. Oxidative stress in chronic renal allograft nephropathy in rats: effects of long-term treatment with carvedilol, BM 91.0228, or alpha-tocopherol. *J Cardiovasc Pharmacol* 2003; 42: 442.
32. Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 1971; 44: 276.

4.4. STUDY IV

LEPTIN AND ADIPONECTIN: BIOMARKERS OF GRAFT DYSFUNCTION?

Fonseca I, Oliveira JC, Santos J, Martins LS, Almeida M, Dias L, Pedroso S, Lobato L, Henriques AC, Mendonça D. Leptin and Adiponectin during the First Week after Kidney Transplantation: Biomarkers of Graft Dysfunction?

(Accepted manuscript in Metabolism – Clinical and Experimental)

Patients with impaired kidney function or chronic kidney disease have increased circulating levels of both pro-inflammatory and anti-inflammatory adipokines that may result from an increase in their systemic production and/or decrease in their renal clearance. Based on this evidence we addressed this study to examine the longitudinal changes of leptin and adiponectin during the first week post-transplant and to test the hypothesis that graft dysfunction would be associated with the accumulation of both adipokines due to their impaired clearance by the kidney. Therefore, we hypothesized that higher levels of leptin and/or adiponectin could serve as early biomarkers of DGF.

The results are summarized as follows:

- Pre-transplant hyperleptinemia was widely prevalent and the serum leptin levels exceeded the upper limit of the normal range in 32 patients (82%). After transplantation, the leptin levels decreased substantially, but at the end of the first week hyperleptinemia was still present in 64% of the patients. Regarding adiponectin, pre-transplant levels over 300 ng/mL were only detected in only 9 patients (22.5%) and following transplantation, the levels of adiponectin were within the normal range.

- The time-course changes of both adipokines were examined according to graft function within the first week after transplantation, and, undoubtedly, the mean levels of leptin were consistently higher in DGF patients at all four timepoints compared to non-DGF recipients; no differences were noted in the mean levels of adiponectin. A linear mixed-effects model was then used to analyze the longitudinal leptin and adiponectin changes, controlling for variables that were found to be associated according to the bivariate analysis (recipient age, gender, BMI and occurrence of DGF) and confirmed the independent association of DGF with the changes of leptinemia but not adiponectinemia during the first week after transplant. The time measurements of leptin, patient gender,

and BMI were the other associated independent factors. According to our estimation, the first mean leptin values after transplantation (day-1) will be approximately two times higher in DGF patients when controlling for the recipient gender and BMI. A significant interaction was observed between the recipient gender and BMI, which was retained in the model, meaning that the effect of BMI on leptin changes is different according to gender, i.e., the effect of an increase in BMI is more pronounced in males and attenuated in females.

- The performance of leptin and adiponectin in discriminating the transplant recipients with DGF was evaluated by ROC analysis, which showed that leptin at day-1 was slightly better than SCr in predicting the need for dialysis within the first week post-transplant (AUC=0.76 for leptin vs. AUC=0.72 for SCr) but adiponectin was not. The results were similar when leptin was adjusted for BMI, but the performance of this adipokine improved considerably in the male gender after splitting the analysis by gender (AUC=0.86).

ARTICLE IN PRESS

METABOLISM CLINICAL AND EXPERIMENTAL XX (2014) XXX–XXX

Available online at www.sciencedirect.com

Metabolism

www.metabolismjournal.com

Leptin and Adiponectin During the First Week After Kidney Transplantation: Biomarkers of Graft Dysfunction?

Isabel Fonseca[a,b,c,*], *José Carlos Oliveira*[d], *Josefina Santos*[a,b], *Jorge Malheiro*[a,b], *La Salete Martins*[a,b], *Manuela Almeida*[a,b], *Leonídio Dias*[a], *Sofia Pedroso*[a,b], *Luísa Lobato*[a,b], *António Castro Henriques*[a,b], *Denisa Mendonça*[e,c]

[a] *Department of Nephrology and Kidney Transplantation, Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal*
[b] *Unit for Multidisciplinary Investigation in Biomedicine (UMIB), Porto, Portugal*
[c] *Institute of Public Health (ISPUP), University of Porto, Porto, Portugal*
[d] *Department of Clinical Chemistry, Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal*
[e] *Department of Population Studies, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal*

ARTICLE INFO

Article history:
Received 9 July 2014
Accepted 3 October 2014

Keywords:
Leptin
Adiponectin
Adipokines
Kidney transplantation
Delayed graft function

ABSTRACT

Context and Objective. Based on evidence that leptin and adiponectin are removed from circulation primarily by the kidney, we designed a study to examine the longitudinal changes of these adipokines during the first week after kidney transplantation (KTx) and to test the hypothesis that higher levels of leptin and/or adiponectin could be early biomarkers of delayed graft function (DGF = dialysis requirement during the first post-transplant week) and acute rejection.

Study Design. Repeated-measures prospective study.

Material and Methods. Forty consecutive adult patients with end-stage renal disease who were undergoing KTx. Leptin and adiponectin were measured in blood samples that were collected before (day-0) and after KTx (days-1, 2, 4 and 7). Linear mixed-models, receiver operating characteristic and area under curve (AUC-ROC) were used.

Results. At post-transplant day-1, leptinemia and adiponectinemia declined 43% and 47%, respectively. At all times studied after KTx, the median leptin levels were significantly higher in patients developing DGF (n = 18), but not adiponectin levels. Shortly after KTx (day-1), leptin values were significantly higher in DGF recipients in contrast to patients with promptly functioning kidneys, approximately two times higher when controlling for gender and BMI. The leptin reduction rate between pre-tranplant and one-day after KTx moderately predicted DGF (AUC = 0.73). On day-1, serum leptin predicted DGF (AUC-ROC = 0.76) with a performance slightly better than serum creatinine (AUC-ROC = 0.72), even after correcting for BMI (AUC-ROC = 0.73). Separating this analysis by gender showed that the performance of leptin in predicting DGF for male gender (AUC-ROC = 0.86) improved.

Abbreviations: AUC-ROC, Area under the receiver-operating characteristic curve; ADPN, Adiponectin; DGF, Delayed graft function; KTx, Kidney transplantation; SCr, Serum creatinine.

* *Corresponding author at:* Department of Nephrology and Kidney Transplantation, Centro Hospitalar do Porto, Hospital de Santo António, Largo Prof. Abel Salazar, 4099–001 Porto, Portugal. Tel.: +351 914740001; fax: +351 223 320 318.
E-mail address: isabelf27@gmail.com (I. Fonseca).

http://dx.doi.org/10.1016/j.metabol.2014.10.003

Please cite this article as: Fonseca I, et al, Leptin and Adiponectin During the First Week After Kidney Transplantation: Biomarkers of Graft Dysfunction?, Metabolism (2014), http://dx.doi.org/10.1016/j.metabol.2014.10.003

Conclusions. Kidney graft function is an independent determinant of leptin levels, but not of adiponectin. Leptin levels at day-1 slightly outperformed serum creatinine in predicting the occurrence of DGF, and more accurately in male gender. No significant association was detected with acute rejection.

1. Introduction

White adipose tissue is now recognized as a multifunctional organ [1]. In addition to its central role in lipid storage, white adipose tissue has a major endocrine function by synthesizing a multitude of protein cytokines termed adipokines. Leptin and adiponectin (ADPN) are two adipokines that elicit generally opposing pro-inflammatory and anti-inflammatory effects [1–4]. In chronic kidney disease, the clinical significance and prognostic implications of leptin and ADPN are not well understood. Patients with chronic kidney disease have increased circulating levels of both adipokines that may result from an increase in their systemic production and/or decrease in their renal clearance [5–13].

The contribution of the kidney in the biodegradation and elimination of leptin and adiponectin appears to be confirmed in studies conducted in kidney transplantation (KTx), where the circulating levels of these adipokines decrease after successful transplantation [14–19]. Based on the growing evidence that leptin and ADPN are removed from the circulation primarily by the kidney, we designed the present study to examine the longitudinal changes of these two adipokines during the first week post-transplant and test the hypothesis that delayed graft function (DGF) would be associated with higher plasma levels of leptin and due to their impaired clearance by the kidney. Therefore, we hypothesized that higher levels of leptin and/or ADPN could serve as early biomarkers of DGF (defined as dialysis requirement within the first week after KTx). Due to the role of leptin in the immune system [9,20–24], the performance of leptin in predicting acute transplant rejection and alloimmunity was also assessed.

2. Patients and Methods

2.1. Study Design and Patient Population

Consecutive patients with end-stage renal disease who were undergoing living or deceased donor KTx between December 2010 and May 2011 were prospectively enrolled. Patients under the age of 18 or who required multiorgan transplants were not included. The Institutional Review Board of Centro Hospitalar do Porto approved the study. Each participant provided informed consent.

2.2. Laboratory Analyses

Blood samples were collected as follows: 3–6 h prior to transplant surgery (pre-transplant); on the following morning, approximately 8–12 h after graft reperfusion (day-1); and then on the second (day-2), fourth (day-4) and seventh days (day-7) after transplant, for a total of five samples per patient.

Serum levels of leptin and ADPN were measured by ELISA based on the direct sandwich technique using kits from Mercodia, Sweden. Standard values of leptin for normal weight people were 2–5.6 ng/mL for male and 3.7–11.1 ng/mL for female. Expected normal values for ADPN were 5–300 ng/mL.

2.3. Definitions

Delayed graft function was defined by the need for dialysis during the first week after KTx.

Acute rejection was defined as either biopsy-proven rejection or anti-rejection treatment without biopsy.

Leptin/BMI ratio was calculated to measure the leptin level while controlling for the BMI contribution.

2.4. Statistical Analyses

The distributions of continuous variables were analyzed using Kolmogorov–Smirnov test and variables showing a positively skewed distribution (leptin and SCr) were natural logarithm transformed prior to parametric test analyses.

Statistical analysis was conducted in three steps. First, a cross-sectional bivariate analysis was performed. Comparisons of continuous variables between groups were carried out using parametric (t-test) or nonparametric (Mann–Whitney) tests; associations between categorical variables were analyzed using the χ^2 test and Fisher's exact test, as appropriate; correlations were assessed using the Pearson or Spearman correlation.

Second, a linear mixed-effects model was used to evaluate the association of DGF with serial changes of leptin (log-transformed) and ADPN, controlling for the recipient's age, gender and BMI.

Third, a receiver operating characteristic (ROC) curve analysis was performed to assess the utility of the levels of leptin and ADPN (as well as SCr) in predicting DGF. The optimal cut-off points were determined.

Statistical analyses were performed using SPSS version 21.0, and a significance level of 0.05 was considered.

3. Results

3.1. Study Cohort

The final study cohort included 40 patients. Their demographic and transplant data are shown in supplementary material (Table S1).

3.2. Leptin and Adiponectin

The time-course of leptin and ADPN levels during the first post-transplant week is summarized in Table 1. Compared to before the transplant, the median leptin levels declined

Please cite this article as: Fonseca I, et al, Leptin and Adiponectin During the First Week After Kidney Transplantation: Biomarkers of Graft Dysfunction?, Metabolism (2014), http://dx.doi.org/10.1016/j.metabol.2014.10.003

Table 1 – Time course of the circulating levels of leptin (ln) and adiponectin within the first post-transplantation week.

		Before KTx	1st Day*	2nd Day	4th Day	7th Day
Leptin (ng/mL)	Overall	15.9 [8.6–31.7]	8.4 [5.3–20.0]	8.0 [3.4–14.7]	8.4 [0.8–25.2]	10.5 [2.9–25.2]
Median [IQR]	DGF	27.4 [12.9–57.7]	12.8 [7.5–22–6]	13.0 [8.7–27.2]	23.1 [1.6–36.7]	16.0 [9.7–26.8]
	Non-DGF	11.6 [5.6–41.2]	5.5 [2.2–10.0]	4.2 [2.4–10.0]	0.8 [6-0–15.6]	1.7 [5.3–23.1]
Adiponectin (ng/mL)	Overall	226 (72)	119 (35)	114 (33)	139 (35)	165 (29)
Mean (S.D)	DGF	250 (80)	110 (39)	105 (37)	130 (39)	157 (32)
	Non-DGF	209 (62)	126 (30)	120 (29)	146 (30)	170 (25)

The values shown are the medians and interquartile ranges or the means and standard deviations;
Abbreviations: KTx = kidney transplantation; DGF = delayed graft function; SD = standard deviation.
* 1st day = 8–12 h after surgery.

significantly at day-1, by approximately 47%, but on day-7, a less-pronounced reduction of approximately 34% was observed. With regard to ADPN, compared to before the transplant, the mean levels of plasma ADPN declined by approximately 47% at day-1 and 27% at day-7.

As expected, the leptin values were positively correlated with BMI at all time points, whereas no correlation was found with ADPN. Levels of leptin, but not ADPN, were significantly higher in female patients, even after adjustment for BMI, and correlated positively with the recipients' ages at all time points. Neither leptin nor ADPN was significantly correlated with time on dialysis, age of the transplant donor, HLA mismatches and cold ischemia time. Additionally, no significant differences were found in relation to the donor status or any type or dose of the immunosuppressive drugs used.

3.3. *Delayed Graft Function and Acute Rejection*

Eighteen (45%) patients had DGF, whereas 22 (55%) patients experienced prompt graft function. Ten recipients had an acute rejection episode during their hospitalization for transplantation, and acute rejection was more frequently diagnosed in patients with DGF than in those with prompt graft function (44% vs. 9%, P = 0.025). Only 2 of the non-DGF patients experienced an acute rejection episode.

3.4. *Longitudinal Changes in the Levels of Leptin and Adiponectin According to Graft Function*

Prior to transplantation, no significant differences were found between patients with DGF or non-DGF with regard to any of the adipokines evaluated. After transplantation, the mean levels of leptin were consistently higher in DGF patients at all time points compared to non-DGF recipients (Table 1 and Fig. 1), whereas no differences were noted in the mean levels of ADPN.

A linear mixed-effects model was used to analyze the longitudinal changes in leptin and ADPN in the two groups of patients (DGF and non-DGF) by controlling for variables found to be associated by the bivariate analysis (recipient age, gender and BMI) and confirmed the independent association of DGF with the longitudinal changes of leptinemia but not adiponectinemia. Regarding leptin, the age of the recipients was no longer statistically significant and was removed from the final model. The time measurements of leptin, patient

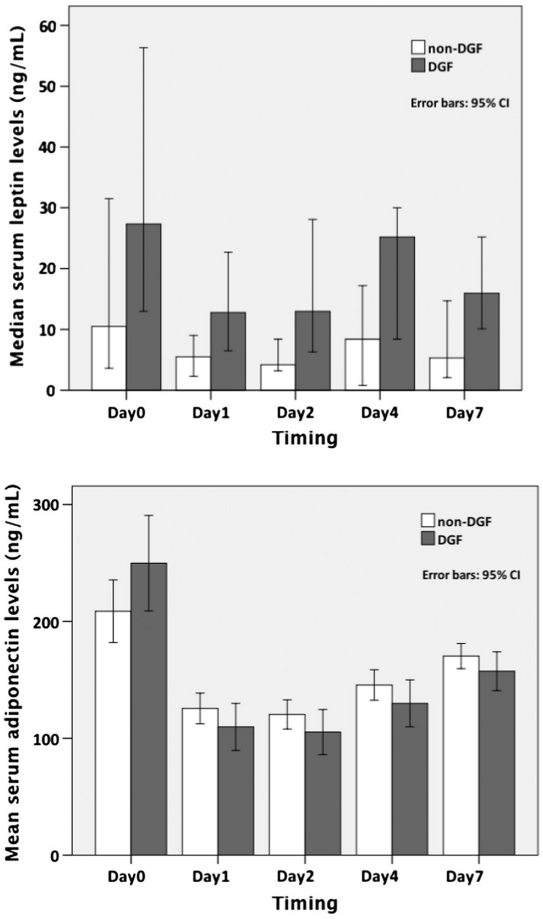
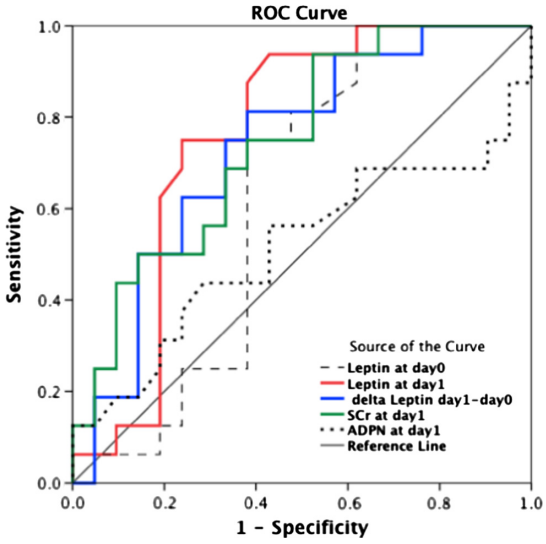


Fig. 1 – Longitudinal changes of the pre-transplant and one-week post-transplant circulating levels of leptin and adiponectin regarding graft function.Evolution of mean and median values of adiponectin and leptin, respectively, with 95% confidence intervals; measurements were performed preoperatively (day 0), and then at first (day 1), second (day 2), fourth (day 4) and seventh (day 7) days after kidney transplantation.

Please cite this article as: Fonseca I, et al, Leptin and Adiponectin During the First Week After Kidney Transplantation: Biomarkers of Graft Dysfunction?, Metabolism (2014), http://dx.doi.org/10.1016/j.metabol.2014.10.003



	AUC (95% CI)	*P* Value
Day 0 (pre-transplant)		
Serum leptin (ng/mL)	0.62 (0.43 -0.81)	0.220
Day 1 (8 to 12 h after surgery)		
Serum leptin (ng/mL)	0.76 (0.60-0.92)	0.008
Serum creatinine (mg/dl)	0.72 (0.59-0.90)	0.012
Serum adiponectin (ng/mL)	0.51 (0.31–0.72)	0.676
Leptin reduction rate (%)		
Δ Leptin$_{\text{day 1-day0}}$(%)	0.73 (0.57-0.90)	0.017

Fig. 2 – Receiver operating characteristic curves for the serum levels of pre-transplant leptin, and leptin, adiponectin and creatinine measured on the first day after KTx, for predicting delayed graft function (DGF). The table lists the areas under the ROC curves of leptin, adiponectin and creatinine for predicting DGF.Abbreviations: SCr = serum creatinine; ADPN = adiponectin; AUC = area under the ROC curve.ΔLeptin$_{\text{day1–day0}}$ = percentage changes in serum leptin between day-1 and day-0.

gender, BMI and DGF were independent factors associated with leptinemia (Table S2). Even after adjusting for recipient gender and BMI, the DGF remained significantly associated with the longitudinal changes of leptinemia: the recipients with prompt graft function presented lower average leptinemia at all times studied. According to our estimation, the first mean leptin values after transplantation will be approximately two times higher in DGF patients when controlling for recipient gender and BMI. A significant interaction was observed between the recipient gender and BMI, which was retained in the model, meaning that the effect of BMI on leptin changes is different according the gender, i.e., the effect of an increase in BMI is more pronounced in males and attenuated in females.

3.5. *Predicting DGF Using Leptin and Adiponectin*

Receiver operating characteristic (ROC) analysis was conducted to evaluate the performance of leptin and ADPN in discriminating the transplant recipients with DGF. This analysis showed that leptin at day-1 was moderately accurate in predicting the need for dialysis within the first week post-transplant, whereas ADPN was not. The area under the curve (A.UC) for the prediction of DGF was 0.76 (P = 0.007; 95% CI: 0.60–0.92) for leptin on day-1, which was slightly better than the diagnostic performance of SCr (AUC = 0.72, P = 0.012; 95% CI: 0.59–0.90) (Fig. 2). When adjusted for BMI (leptin/BMI ratio), the performance of one-day leptin in predicting DGF was similar (AUC = 0.73, P = 0.017; 95% CI: 0.57–0.90). The potential of pre-transplant leptin levels (day-0) in predicting DGF was also assessed due to higher levels of leptin in DGF vs. non-DGF patients before KTx, but this variable performed poorly having a non-significant AUC of 0.62.

An ROC curve was also created to evaluate the performance of the reduction in serum leptin levels between pre-transplant and day-1 (Δleptin $_{\text{day 1–day0}}$) in predicting DGF and resulted in an AUC of 0.73 comparable to one-day SCr (Fig. 2). In analyzing the ROC curve of Δleptin $_{\text{day 1–day0}}$, the optimal sensitivity and specificity were achieved at a leptin reduction ratio of 41.7% (sensitivity: 81%; specificity: 62%; positive and negative predictive values of 68 and 77, respectively).

In analyzing the ROC curve of leptin on day-1, the optimal sensitivity and specificity were achieved at a leptin concentration of 10.8 ng/mL (sensitivity: 73%; specificity: 77%; positive and negative predictive values of 76 and 74, respectively). Separate analysis by gender, showed that the performance of leptin at day-1 in diagnosing DGF improved considerably for male gender (AUC = 0.86, P = 0.004; 95% CI: 0.70–1.00), and the optimal sensitivity and specificity were achieved at a leptin concentration of 6.4 ng/mL (sensitivity: 89%; specificity: 79%; positive and negative predictive values of 81 and 88, respectively). Due to small number of female recipients (n = 14), this analysis was not done separately for female gender.

3.6. *Predicting Acute Rejection and Post-Transplant Anti-HLA Antibodies Using Leptin*

The predictive power of serum leptin in acute rejection was also analyzed, but no significant ability to predict acute rejection was found at any time-point considered. Pre-transplant anti-HLA screening was positive in 9 patients (22.5%) and in 12 patients (30%) during the first year following KTx; in 2 patients (5.0%) they were detected before transplant only, in 5 patients (12.5%) after transplant only, and in 7 patients (17.5%) both before and after transplantation. No significant association between leptin levels and anti-HLA positivity was showed.

4. Discussion

To date, most of the research on leptin and ADPN has been focused on its association with metabolic and cardiovascular health. Up to know, no reported study has examined the clinical utility of these adipokines in the diagnosis of graft dysfunction after KTx. We studied the performance of leptin and ADPN levels in predicting DGF using ROC analysis and compared these results with the routinely used SCr. Leptin

Please cite this article as: Fonseca I, et al, Leptin and Adiponectin During the First Week After Kidney Transplantation: Biomarkers of Graft Dysfunction?, Metabolism (2014), http://dx.doi.org/10.1016/j.metabol.2014.10.003

levels at day-1, but not ADPN, slightly outperformed the traditional biomarker SCr in assessing the prognosis of DGF. Contrary to our expectations, leptinemia did not add substantially to the early discriminative or predictive power of SCr for the detection of DGF. Although degradation pathways of leptin have not been clearly elucidated, it has been suggested that endogenous leptin is rapidly cleared from circulation by glomerular filtration and by renal uptake and degradation [6,25–27]. However, it is possible that other factors that coexist in the immediate post-transplant period might stimulate leptin synthesis and delay its elimination.

Inflammation [28] has been implicated in augmenting leptin secretion. Surgical stress is also associated with an increase in serum leptin concentration [29,30]. Both of these conditions concur in KTx. Kidney transplantation is a surgical procedure that involves an inevitable ischemia-reperfusion injury with consequent deleterious activation of cellular oxidases causing oxidative damage, tissue injury and inflammation [31]. Leptin is an acute phase reactant that is involved in the cytokine network of acute inflammation and stress response [29]. It is possible that inflammatory cytokines resulting from the organ transplant process can stimulate leptin synthesis and attenuate its clearance from circulation, mainly in recipients with graft dysfunction. This would also explain why the decrement of plasma leptin concentration in the immediate post-transplant period did not reach normal levels in most of patients, even in those with prompt graft function. At day-7, 64% of our recipients still had levels that exceeded the upper limit of leptin reference range, or 40% considering only the recipients with prompt graft function. Some other studies show the maintenance of elevated serum concentrations of leptin in KTx recipients [32,33].

This study was designed based upon the assumption that impaired clearance of leptin (and ADPN) could signal graft dysfunction earlier than SCr. Of the two adipokines measured, leptin most closely fulfilled our initial hypothesis. Adiponectinemia was not significantly higher in recipients with graft dysfunction and was not a predictor of DGF. At least during the first week, graft dysfunction did not reflect impaired clearance of ADPN, suggesting that factors other than renal function may be involved. A study from Song and coworkers [34] demonstrated a decline in circulating ADPN levels during the initial 72 h after a subtotal nephrectomy in mice with renal failure, associated to down regulation of ADPN. Following this reasoning, we can also speculate that the decrease of circulating ADPN levels observed within the first week after KTx could be due to two different mechanisms according to graft function: enhanced filtration of circulating ADPN and urinary excretion in prompt graft patients; and decline in local expression of ADPN in glomerular endothelium as a result of amplified ischemia-reperfusion injury that usually describes DGF.

Ischemia/reperfusion injury is undoubtedly an important variable that can influence the outcome of the transplanted kidney since it is a major risk factor for the development of DGF and acute rejection [35,36]. In fact, there is an apparent synergy between the initial injuries of ischemia/reperfusion and acute graft rejection [37], and because of innate immune response this deleterious condition can lead to graft dysfunction [38]. Hence, the immune response against a transplanted organ may not solely involve a major histocompatibility complex specific alloimmune response, but in addition, an immediate nonspecific inflammatory response caused by ischemia/reperfusion injury [39]. Recent studies highlight the role of leptin in the immune system [9,20–24], therefore and beyond DGF we assessed leptin ability to predict acute rejection and anti-HLA antibodies following KTx. Possibly because of the small number of patients with acute rejection and anti-HLA antibodies no significant predictive value was found. Few studies [22] have addressed the influence of leptin, or other adipose tissue-derived products, on the allograft response and outcome, and to the best of our knowledge none in KTx.

In summary, the findings from the present study clearly demonstrate the importance of graft function in the clearance of leptin from the circulation, but not that of ADPN. Graft function was a stronger determinant of leptinemia, and the levels of this adipokine slightly outperformed SCr in predicting DGF. The maintenance of elevated levels of leptin in KTx and the role of this adipokine in allo-immunity are some of the questions that arise from this study, showing that much is still unknown in this field [40].

Funding Sources

None.

Declaration of Interest

The authors declare no conflict of interest regarding the publication of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.metabol.2014.10.003.

REFERENCES

[1] Sahin-Efe A, Katsikeris F, Mantzoros CS. Advances in adipokines. Metabolism 2012;61:1659–65.
[2] Wannamethee SG, Tchernova J, Whincup P, Lowe GD, Kelly A, Rumley A, et al. Plasma leptin: associations with metabolic, inflammatory and haemostatic risk factors for cardiovascular disease. Atherosclerosis 2007;191:418–26.
[3] Nakamura K, Fuster JJ, Walsh K. Adipokines: a link between obesity and cardiovascular disease. J Cardiol 2014; 63:250–9.
[4] Van de Voorde J, Pauwels B, Boydens C, Decaluwe K. Adipocytokines in relation to cardiovascular disease. Metabolism 2013;62:1513–21.
[5] Becker B, Kronenberg F, Kielstein JT, Haller H, Morath C, Ritz E, et al. Renal insulin resistance syndrome, adiponectin and cardiovascular events in patients with kidney disease: the mild and moderate kidney disease study. J Am Soc Nephrol 2005;16:1091–8.

Please cite this article as: Fonseca I, et al, Leptin and Adiponectin During the First Week After Kidney Transplantation: Biomarkers of Graft Dysfunction?, Metabolism (2014), http://dx.doi.org/10.1016/j.metabol.2014.10.003

[6] Cumin F, Baum HP, Levens N. Leptin is cleared from the circulation primarily by the kidney. Int J Obes Relat Metab Disord 1996;20:1120–6.
[7] Kataoka H, Sharma K. Renal handling of adipokines. Contrib Nephrol 2006;151:91–105.
[8] Komura N, Kihara S, Sonoda M, Maeda N, Tochino Y, Funahashi T, et al. Increment and impairment of adiponectin in renal failure. Cardiovasc Res 2010;86:471–7.
[9] Moraes-Vieira PM, Bassi EJ, Araujo RC, Camara NO. Leptin as a link between the immune system and kidney-related diseases: leading actor or just a coadjuvant? Obes Rev 2012; 13:733–43.
[10] Saraheimo M, Forsblom C, Fagerudd J, Teppo AM, Pettersson-Fernholm K, Frystyk J, et al. Serum adiponectin is increased in type 1 diabetic patients with nephropathy. Diabetes Care 2005;28:1410–4.
[11] Shoji T, Nishizawa Y, Emoto M, Maekawa K, Hiura Y, Tanaka S, et al. Renal function and insulin resistance as determinants of plasma leptin levels in patients with NIDDM. Diabetologia 1997;40:676–9.
[12] Zoccali C, Mallamaci F, Tripepi G. Adipose tissue as a source of inflammatory cytokines in health and disease: focus on end-stage renal disease. Kidney Int Suppl 2003:S65–8.
[13] Ziegelmeier M, Bachmann A, Seeger J, Lossner U, Kratzsch J, Bluher M, et al. Adipokines influencing metabolic and cardiovascular disease are differentially regulated in maintenance hemodialysis. Metabolism 2008;57:1414–21.
[14] Chudek J, Adamczak M, Karkoszka H, Budzinski G, Ignacy W, Funahashi T, et al. Plasma adiponectin concentration before and after successful kidney transplantation. Transplant Proc 2003;35:2186–9.
[15] Kokot F, Adamczak M, Wiecek A. Plasma leptin concentration in kidney transplant patients during the early post-transplant period. Nephrol Dial Transplant 1998;13:2276–80.
[16] Landt M, Brennan DC, Parvin CA, Flavin KS, Dagogo-Jack S, Coyne DW. Hyperleptinaemia of end-stage renal disease is corrected by renal transplantation. Nephrol Dial Transplant 1998;13:2271–5.
[17] Kayacan SM, Yildiz A, Kazancioglu R, Sahin S, Sever MS, Ark E. The changes in serum leptin, body fat mass and insulin resistance after renal transplantation. Clin Transplant 2003; 17:63–8.
[18] El Haggan W, Chauveau P, Barthe N, Merville P, Potaux L, Aparicio M. Serum leptin, body fat, and nutritional markers during the six months post-kidney transplantation. Metabolism 2004;53:614–9.
[19] Souza GC, Costa C, Scalco R, Goncalves LF, Manfro RC. Serum leptin, insulin resistance, and body fat after renal transplantation. J Ren Nutr 2008;18:479–88.
[20] Paz-Filho G, Mastronardi C, Franco CB, Wang KB, Wong ML, Licinio J. Leptin: molecular mechanisms, systemic pro-inflammatory effects, and clinical implications. Arq Bras Endocrinol Metabol 2012;56:597–607.
[21] Conde J, Scotece M, Gomez R, Lopez V, Gomez-Reino JJ, Lago F, et al. Adipokines: biofactors from white adipose tissue. A complex hub among inflammation, metabolism, and immunity. Biofactors 2011;37:413–20.
[22] Moraes-Vieira PM, Bassi EJ, Larocca RA, Castoldi A, Burghos M, Lepique AP, et al. Leptin deficiency modulates allograft survival by favoring a Th2 and a regulatory immune profile. Am J Transplant 2013;13:36–44 [corrected].
[23] Moraes-Vieira PM, Larocca RA, Bassi EJ, Peron JP, Andrade-Oliveira V, Wasinski F, et al. Leptin deficiency impairs maturation of dendritic cells and enhances induction of regulatory T and Th17 cells. Eur J Immunol 2014;44:794–806.
[24] De Rosa V, Procaccini C, Cali G, Pirozzi G, Fontana S, Zappacosta S, et al. A key role of leptin in the control of regulatory T cell proliferation. Immunity 2007;26:241–55.
[25] Meyer C, Robson D, Rackovsky N, Nadkarni V, Gerich J. Role of the kidney in human leptin metabolism. Am J Physiol 1997; 273:E903–7.
[26] Cumin F, Baum HP, Levens N. Mechanism of leptin removal from the circulation by the kidney. J Endocrinol 1997;155: 577–85.
[27] Zeng J, Patterson BW, Klein S, Martin DR, Dagogo-Jack S, Kohrt WM, et al. Whole body leptin kinetics and renal metabolism in vivo. Am J Physiol 1997;273:E1102–6.
[28] Maury E, Brichard SM. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol Cell Endocrinol 2010;314:1–16.
[29] Chachkhiani I, Gurlich R, Maruna P, Frasko R, Lindner J. The postoperative stress response and its reflection in cytokine network and leptin plasma levels. Physiol Res 2005;54:279–85.
[30] Hernandez C, Simo R, Chacon P, Sabin P, Baena JA, Castellanos JM, et al. Influence of surgical stress and parenteral nutrition on serum leptin concentration. Clin Nutr 2000;19:61–4.
[31] Eltzschig HK, Eckle T. Ischemia and reperfusion—from mechanism to translation. Nat Med 2011;17:1391–401.
[32] Kokot F, Adamczak M, Wiecedil A, Spiechowicz U, Mesjasz J. Plasma immunoreactive leptin and neuropeptide Y levels in kidney transplant patients. Am J Nephrol 1999;19: 28–33.
[33] Kagan A, Haran N, Leschinsky L, Sarafian R, Aravot D, Dolberg J, et al. Serum concentrations of leptin in heart, liver and kidney transplant recipients. Isr Med Assoc J 2002;4:213–7.
[34] Song Y, Yu Q, Zhang J, Huang W, Liu Y, Pei H, et al. Increased myocardial ischemia-reperfusion injury in renal failure involves cardiac adiponectin signal deficiency. Am J Physiol Endocrinol Metab 2014;306:E1055–64.
[35] Kosieradzki M, Rowinski W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. Transplant Proc 2008;40:3279–88.
[36] Ponticelli C. Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. Nephrol Dial Transplant 2014;29(6):1134–40.
[37] Dragun D, Lukitsch I, Tullius SG, Qun Y, Park JK, Schneider W, et al. Inhibition of intercellular adhesion molecule-1 with antisense deoxynucleotides prolongs renal isograft survival in the rat. Kidney Int 1998;54:2113–22.
[38] Li L, Okusa MD. Macrophages, dendritic cells, and kidney ischemia-reperfusion injury. Semin Nephrol 2010;30:268–77.
[39] Koo DD, Welsh KI, Roake JA, Morris PJ, Fuggle SV. Ischemia/reperfusion injury in human kidney transplantation: an immunohistochemical analysis of changes after reperfusion. Am J Pathol 1998;153:557–66.
[40] Modan-Moses D, Paret G. Leptin and transplantation: pieces are still missing in the puzzle. Isr Med Assoc J 2002;4:207–8.

Please cite this article as: Fonseca I, et al, Leptin and Adiponectin During the First Week After Kidney Transplantation: Biomarkers of Graft Dysfunction?, Metabolism (2014), http://dx.doi.org/10.1016/j.metabol.2014.10.003

4.5. STUDY V

COMBINING BIOMARKERS IN KIDNEY TRANSPLANTATION

Fonseca I, Reguengo H, Oliveira JC, Martins LS, Malheiro J, Almeida M, Santos J, Dias L, Pedroso S, Lobato L, Henriques AC, Mendonça D. A Triple-Biomarker Approach for the Detection of Delayed Graft Function using Serum Creatinine, Cystatin C, and Malondialdehyde

(Submitted)

One biomarker can be very adept at diagnosing a disease, but the combined power of two or more biomarkers can be even better. Newly introduced biomarkers should complement and have incremental diagnostic and prognostic value over and above the current established markers. Utilizing the same prospective cohort, our primary goal for the current study was to investigate the value of combining these new markers with the widely used SCr level in predicting DGF.

- Based on the previous research focused on single biomarkers for DGF diagnosis, this study identified a simple and clinically applicable tool for detecting graft dysfunction earlier than SCr alone.
- A triple-biomarker approach, using SCr, CysC, and MDA measured 8 to 12 h after Kidney transplantation, was the most informative combination, resulting in an increased ability (AUC=0.96) to distinguish patients with graft damage and those who would require dialysis within the first week.
- A formula was achieved by fitting a multiple logistic regression model for combining SCr, MDA, and CysC measured 8 to 12 hours after kidney transplantation. Using this formula and calculating the predicted values, the optimal sensitivity and specificity occurred at a value of 0.278 (sensitivity: 100%; specificity: 86%; positive and negative predictive value: 88% and 100%, respectively; and positive and negative likelihood ratios of 7.35 and 0, respectively).
- Combining biomarkers from different pathophysiologic pathways seems to be rational and a reliable strategy for optimizing sensitivity and specificity and obtaining additive diagnostic and prognostic information.

A TRIPLE-BIOMARKER APPROACH FOR THE DETECTION OF DELAYED GRAFT FUNCTION AFTER KIDNEY TRANSPLANTATION USING SERUM CREATININE, CYSTATIN C, AND MALONDIALDEHYDE

Isabel Fonseca[1,2,3], Henrique Reguengo[4], José Carlos Oliveira[4], La Salete Martins[1,2], Jorge Malheiro[1,2], Manuela Almeida[1,2], Josefina Santos[1,2], Leonídio Dias[1], Sofia Pedroso[1], Luísa Lobato[1,2], António Castro Henriques[1,2], and Denisa Mendonça[5,3]

(***Manuscript submitted***)

Authors' Affiliations

[1] Department of Nephrology and Kidney Transplantation, Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal

[2] Unit for Multidisciplinary Investigation in Biomedicine (UMIB), Porto, Portugal

[3] Institute of Public Health (ISPUP), University of Porto, Porto, Portugal

[4] Department of Clinical Chemistry, Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal

[5] Department of Population Studies, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal

ABSTRACT

Serum creatinine (SCr) alone does not allow for the early diagnosis of delayed graft function (DGF) following kidney transplantation (KTx). The diagnostic utility of urinary neutrophil gelatinase-associated lipocalin (uNGAL), serum leptin, malondialdehyde (MDA), and cystatin C (CysC) for the early detection of DGF was previously evaluated by our group in a prospective cohort study of 40 consecutive adults undergoing KTx. Because no single biomarker achieved adequate sensitivity or specificity for practical purposes, this study was designed to evaluate the combined use of new markers with SCr. Urine and blood samples were collected 8-to-12 h after KTx (day-1). Logistic regression was used to combine the biomarkers, and receiver operating characteristic curves and areas under the curve (AUC-ROC) were generated.

Eighteen recipients developed DGF (dialysis requirement during the first post-transplant week). On day-1, the AUC for SCr to predict DGF was 0.73, 0.88 for uNGAL, 0.90 for MDA, 0.76 for leptin, and 0.91 for CysC. Adding new biomarkers to SCr enhanced the performance of DGF prediction, and the best combination was achieved with SCr, MDA, and CysC (AUC=0.96, sensitivity=100%; specificity=86%). A combination of graft damage biomarkers outperformed SCr in the early diagnosis of DGF, and the best performance was achieved by a triple-marker approach, using SCr, MDA, and CysC.

A TRIPLE-BIOMARKER APPROACH FOR THE DETECTION OF DELAYED GRAFT FUNCTION AFTER KIDNEY TRANSPLANTATION USING SERUM CREATININE, CYSTATIN C, AND MALONDIALDEHYDE

Isabel Fonseca[1,2,3], Henrique Reguengo[4], José Carlos Oliveira[4], La Salete Martins[1,2], Jorge Malheiro[1,2], Manuela Almeida[1,2], Josefina Santos[1,2], Leonídio Dias[1], Sofia Pedroso[1], Luísa Lobato[1,2], António Castro Henriques[1,2], and Denisa Mendonça[5,3]

(***Manuscript submitted***)

INTRODUCTION

Delayed graft function (DGF) is a clinical diagnosis that describes kidney dysfunction immediately after kidney transplantation (KTx), usually related to ischemic damage to the graft. The rate of DGF after KTx varies from 2% to 50%, depending on the definition and the center's practice, and it is one of the most important risk factors for both acute rejection and impaired renal function at one year [1-4]. Ischemia/reperfusion injury after organ transplantation is a major cause of DGF, which is associated with prolonged hospital stay, additional invasive procedures, supplementary costs and greater risk of early and long-term graft loss. Much research effort has been devoted to the search for DGF predictors, also called markers, that would allow for early determination of allograft function and of prognoses for one-year and long-term graft survival. Unfortunately, the use of baseline characteristics alone (donor and recipient age, pre-transplant dialysis time, HLA mismatches, etc.) has limited accuracy in predicting both early and late graft outcomes. Several clinical algorithms have been proposed to predict DGF based on pre-operative risk factors, but none has been used routinely in clinical practice [5-7].

Currently, although inadequate for timely diagnosis and prognosis, serum creatinine (SCr) is an established and most commonly used clinical indicator of kidney function [8]. Recent insights have suggested that allograft injury and recovery could be better characterized by new biomarkers. In KTx, numerous biomarkers have been evaluated over the past decade, but thus far, the evidence to support their use in routine practice has been limited [9-12]. Newly introduced biomarkers should complement and have incremental diagnostic and prognostic value over and above the current established markers. It would be ideal to discover a single marker with very high discriminatory accuracy, defined in terms of test sensitivity and specificity [13, 14]. However, for most diseases, and particularly for KTx, single markers might not reflect all of the facets of initial graft dysfunction, and they do not have adequate sensitivity or specificity for practical purposes. One approach to increasing the clinical value of biomarkers and improving their screening sensitivity is to identify additional biomarkers and to combine them because a multimarker strategy might better characterize the complexity of DGF [15].

We recently demonstrated, in a prospective cohort study, that urinary neutrophil gelatinase-associated lipocalin (uNGAL) [16], serum cystatin C (CysC) and malondialdehyde (MDA) [17], and serum leptin (ongoing study) within the first 24 h following KTx predicted DGF better than SCr levels or changes in SCr levels. Utilizing the same cohort, our primary goal for the current study was to investigate the value of combining these new markers with the widely used SCr level in predicting DGF (defined as dialysis requirement within the first week after KTx).

PATIENTS AND METHODS

Study Design and Patient Population

Consecutive patients with end-stage renal disease, who were undergoing living or deceased donor KTx in the Department of Nephrology and Kidney Transplantation of Centro Hospitalar do Porto between December 2010 and May 2011, were prospectively enrolled. Patients younger than the age of 18 years old or who required multiorgan transplants were not included. After transplantation, recipients with primary graft failure were excluded. The Institutional Review Board of Centro Hospitalar do Porto approved this study. Each participant provided informed consent.

Sample Collection and Biomarker Measurements

Blood and urinary samples were collected as follows: 3 to 6 h prior to transplant surgery (pre-transplant); on the following morning, approximately 8 to 12 h after graft reperfusion (day-1); and then on the second, fourth, and seventh days after transplant, for a total of five samples per patient. All samples were immediately centrifuged, aliquoted and frozen within one hour after collection. Subsequently, they were stored at -80°C until analysis, which was performed approximately one to two weeks after collection.

The candidate biomarkers were measured using the following methods. *Neutrophil gelatinase-associated lipocalin* was measured in urine (uNGAL) using a two-step chemiluminescent microparticle immunoassay on a standardized clinical platform (ARCHITECT, Abbott Diagnostics, Germany). *Malondialdehyde* was measured in plasma using a commercial high-performance liquid chromatography kit (Chromsystems, Munich, Germany). *Leptin* was measured in serum by ELISA, based on the direct sandwich technique using kits from Mercodia (Sweden). *Cystatin C* was measured in serum (CysC) by particle-enhanced nephelometric immunoassay (Siemens Diagnostics, Germany).

Definitions

Delayed graft function was defined by the need for dialysis during the first week after KTx. "*Prompt*" function (non-DGF) was characterized by no dialysis session being required

during the first post-transplantation week.

Percentage change in SCr (△SCr*)* was calculated individually for each subject as the difference between SCr on day-1 and day-0 divided by SCr on day-0 (or difference between day-2 and day-1 divided by SCr on day-1), multiplied by 100.

Statistical Analyses

The distributions of continuous variables were analyzed, and the Kolmogorov-Smirnov test was performed to assess their deviation from the normal distribution. Variables showing a positively skewed distribution, such as SCr, uNGAL, and serum leptin, were natural logarithm transformed (ln) prior to parametric test analyses. Normally distributed variables are presented as the mean values and standard deviations (SDs), and variables exhibiting skewed distributions are presented as the medians and 25^{th}-75^{th} quartiles (IQR=interquartile range). Categorical variables are reported as percentages.

Correlations between biomarkers were assessed using Pearson's correlation coefficient, and SCr, uNGAL, and serum leptin levels were log-transformed before analysis to normalize the distribution.

Receiver-operating characteristics (ROC) curves were generated to analyze single and multiple biomarkers, and the areas under the curve (AUCs) were calculated. For joint analysis of multiple biomarkers, a fitted multiple logistic regression model (with DGF as the dependent variable) was used to yield maximum sensitivity and specificity. Pre-transplant variables known to be associated with DGF and considered potential confounders were included in the models. Multicollinearity among covariates was examined through the correlations of regression coefficients. The optimal cut-off points were determined by the largest sum of sensitivity and specificity, and the positive and negative likelihood ratios were calculated as follows: sensitivity/(1–specificity) and (1–sensitivity)/specificity, respectively [18].

Statistical analyses were performed using the SPSS software, version 22.0, and a significance level of 0.05 was considered.

RESULTS

During the recruitment period, 42 patients were enrolled consecutively. Two recipients had primary graft failure and were excluded. Therefore, the final study cohort included 40 patients. Their demographic and transplant data are shown in Table 1.

Eighteen (45%) patients had DGF, whereas 22 (55%) patients experienced prompt graft function. The rate of DGF was higher in kidney grafts from deceased donors, but this difference was not statistically significant (51.7% vs. 27.3%, *P*=0.286). In terms of the traditional predictors of DGF, except for cold ischemia time and recipient age, no

significant differences were found between the DGF and non-DGF groups regarding the baseline characteristics or induction therapy (Table 1).

Table 1. Summary of the demographic and clinical characteristics of the kidney transplant donors and recipients (total patient cohort and separated into delayed or prompt graft function)

	TOTAL (n=40)	DGF (n=18)	NON-DGF (n=22)	*P-VALUE*
DONOR				
Age (yr)	51.2 ± 11.4	51.1 ± 13.4	51.2 ± 9.9	0.172
Male sex	26 (65)	14 (78)	12 (54.5)	0.125
Living donor	11 (27.5)	3 (16.7)	8 (36.4)	0.165
Expanded criteria donors	3 (7.5)	1 (5.6)	2 (9.1)	0.541
Serum creatinine (mg/dl)	0.81 ± 0.18	0.85 ± 0.21	0.78 ± 0.16	0.318
DONOR-RECIPIENT				
HLA mismatches	3.39 ± 1.24	3.38 ± 1.07	3.41 ± 1.46	0.941
Cold ischemia time (h)	12.1 ± 7.9	15.2 ± 7.8	9.6 ± 7.3	0.035*
Living donor	2.8 ± 0.5	2.5 ± 0.5	3.0 ± 0.5	0.204
Deceased donor	16.2 ± 5.9	18.1 ± 5.1	14.1 ± 6.2	0.088
RECIPIENT				
Age (yr)	49.2 ± 15.2	56.3 ± 10.9	43.3 ± 15.9	0.006*
Male sex	26 (65)	11 (61)	15 (68)	0.641
Caucasian	40 (100)	18 (100)	22 (100)	-
BMI (kg/m^2)	24.8 ± 4.9	26.2 ± 4.4	23.6 ± 5.0	0.091
Previous kidney transplant	2 (5)	0 (0)	2 (9.1)	
Time on dialysis (yr)	4.4 ± 4.7	5.6 ± 6.2	3.4 ± 2.3	0.135
Pre-transplant therapy				
Dialysis	38 (95)	18 (100)	20 (90.9)	0.296
Pre-emptive transplantation	2 (5)	0 (0)	2 (9.1)	
Cause of kidney disease				
IgA nephropathy	7 (17.5)	2 (11.1)	5 (22.7)	-
Glomerulonephritis	6 (15.0)	4 (22.2)	2 (9.1)	-
Diabetic nephropathy	5 (12.5)	3 (16.7)	2 (9.1)	-
Autosomal-dominant polycystic kidney disease	3 (7.5)	3 (16.7)	0 (0)	-
Unknown	4 (10.0)	1 (5.6)	3 (13.6)	-
Others	15 (37.5)	5 (27.8)	10 (45.5)	-
Peak PRA (%)	5.5 ± 15.1	5.0 ± 15.0	5.9 ± 15.5	0.853
0	29 (72.5)	14 (77.8)	15 (68.2)	-
1-25	8 (20.0)	3 (16.7)	5 (22.7)	-
26-75	3 (7.5)	1 (5.6)	2 (9.0)	-
Current PRA (%)	2.3 ± 8.6	3.1 ± 11.7	1.6 ± 4.9	0.585
0	34 (85)	15 (83.3)	19 (86.4)	-
1-25	5 (12.5)	2 (11.1)	3 (13.6)	-
26-50	1 (2.5)	1 (5.6)	0 (0)	-
Induction regimen				
Antithymocyte globulin (ATG-F)	4 (10)	1 (5.6)	3 (13.6)	0.613
Basiliximab/daclizumab	30 (75)	14 (77.8)	16 (72.7)	0.789
Immunosuppression at the time of discharge				
Steroids	38 (95.0)	18 (100)	20 (90.9)	0.296
Tacrolimus	38 (95.0)	17 (94.4)	21 (95.5)	0.886
Cyclosporine A	2 (0.05)	1 (5.6)	1 (5.6)	0.884

Values are expressed as means ± standard deviations or as absolute numbers and percentages. Comparisons of continuous variables between groups were analyzed using parametric (t-test) or nonparametric (Mann-Whitney) tests; the associations between categorical variables were analyzed using the $\chi 2$ test and Fisher's exact test.
Abbreviations: HLA, human leukocyte antigen; BMI, body mass index; PRA, panel reactive antibody.

Candidate biomarkers for the detection of DGF

Detailed results regarding each biomarker were previously reported [16, 17]. Briefly, levels of uNGAL, serum MDA, leptin and CysC were significantly higher in DGF patients. Longitudinal changes in the four biomarkers, according to graft function within the first week after KTx, are shown in Figure 1. The correlations between markers are displayed in table 2. All of the biomarkers were positively correlated, with the exception of leptin.

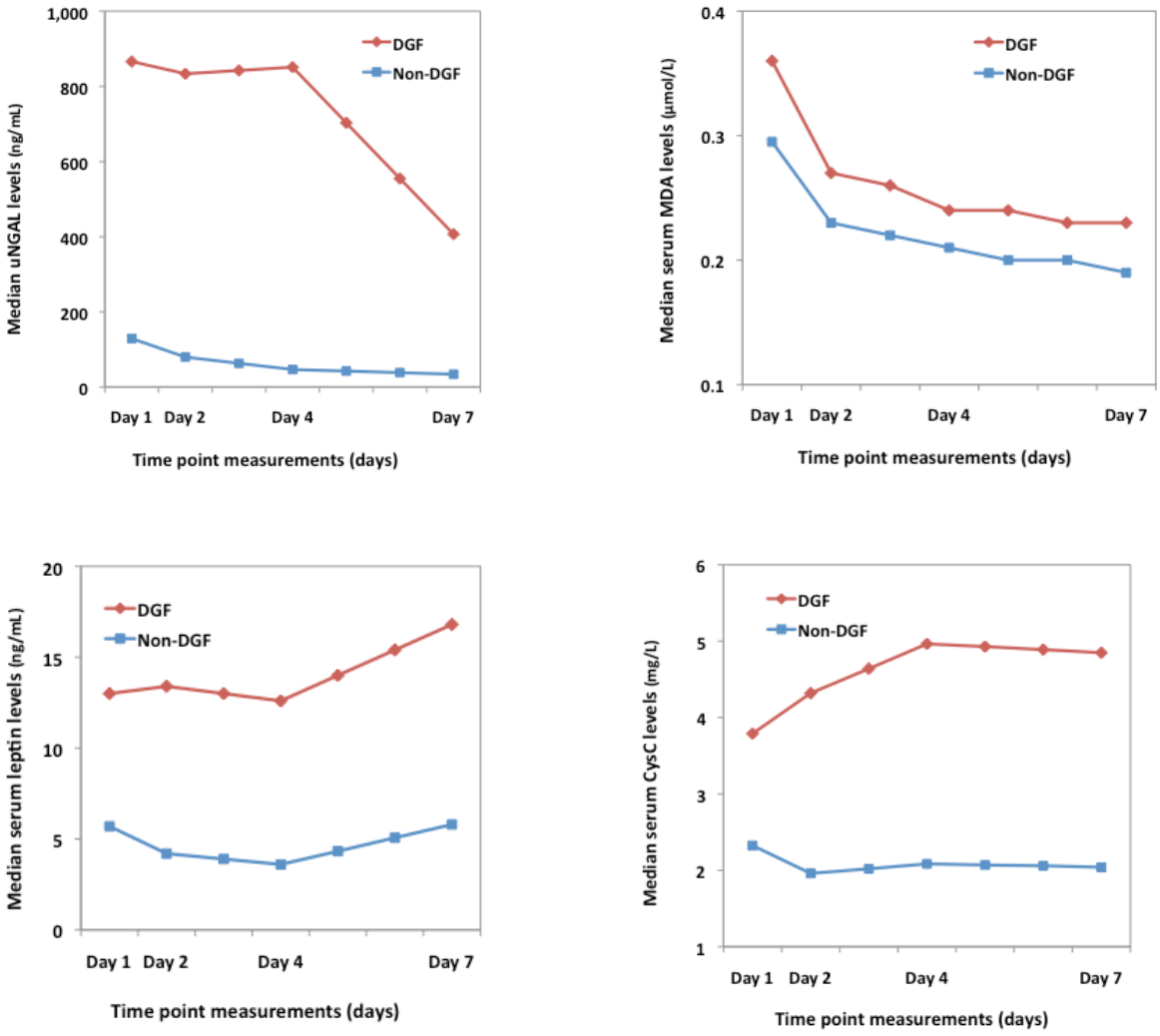


Figure 1. Longitudinal changes in the four biomarkers during the first week after kidney transplantation according to graft function. Graphs show median levels of urinary neutrophil gelatinase-associated lipocalin (uNGAL), serum malondialdehyde, serum leptin, and serum cystatin C (CysC) at multiple time points and according to the occurrence or not of delayed graft function (DGF and non-DGF, respectively).

Table 2. Correlation between markers measured on the first day after kidney transplantation

	SCr (ln)	$\triangle SCr_{day1-day0}$	$\triangle SCr_{day2-day1}$	uNGAL (ln)	MDA	Leptin (ln)	CysC
SCr (ln)	-	0.17	0.07	0.55*	0.45*	-0.10	0.51*
$\triangle SCr_{day1-day0}$	0.17	-	0.42*	0.49	0.46*	0.02	0.60**
$\triangle SCr_{day2-day1}$	0.07	0.42*	-	0.63**	0.32†	0.16	0.45*
uNGAL (ln)	0.55*	0.49*	0.63**	-	0.52*	0.25	0.80**
MDA	0.45*	0.46*	0.32†	0.52*	-	0.12	0.58**
Leptin (ln)	-0.10	0.02	0.16	0.25	0.12	-	0.15
CysC	0.51*	0.60**	0.45*	0.80**	0.58**	0.15	-

Values are given as Pearson's coefficients and relevant *P*-values. †P<0.05; *P<0.01; **P<0.001. Logarithm-transformed values of SCr, uNGAL, and leptin were used for analysis.
Abbreviations: SCr = serum creatinine; uNGAL = urinary neutrophil gelatinase-associated lipocalin; MDA = serum malondialdehyde; CysC = serum cystatin C; ln = logarithm
$\triangle SCr_{day1-day0}$ and $\triangle SCr_{day2-day1}$ = percentage changes in SCr between day-1 and day-0 and between day-2 and day-1.

ROC analysis of day-1 SCr, uNGAL, and serum CysC, MDA, and leptin, individually and combined as early biomarkers for DGF detection

Receiver operating characteristic curves were generated, and AUCs were calculated to estimate the diagnostic accuracy of each marker for DGF. Based on the logistic models, the predicted values of the combined predictors were calculated and analyzed using ROC curves. The performance of day-1 SCr alone and in association with each of the new markers (uNGAL, serum CysC, MDA, and leptin) in diagnosing DGF is illustrated in Table 3. On the morning following KTx, the AUCs for the diagnosis of DGF using only SCr or △SCr were 0.732 and 0.755. When adding each of the new markers to SCr, the performance of the combined markers improved substantially over that of the SCr individually, and the AUCs were 0.914 for CysC, 0.902 for MDA, 0.878 for uNGAL, and 0.792 for leptin. Combining SCr with the four new markers, the AUC was 0.95, and the best diagnostic performance was for SCr combined with serum CysC and MDA (AUC=0.96).

After including SCr, CysC, and MDA in a logistic model for combining these markers, none of the pre-transplant variables traditionally associated with DGF, namely donor and recipient age, pre-transplant dialysis time, HLA mismatches, and cold ischemia time, were statistically significant, and they were removed from the final model by backward elimination (Table 4). Recipient and donor age were included in the models separately, due to multicollinearity observed when both variables were included. For the same reason, given the multicollinearity when pre-transplant time on dialysis and cold ischemia time were entered into the model, time on dialysis was included, and donor status was used instead of cold ischemia time.

Table 3. Areas under the curve (AUCs) for single and combined biomarkers measured on the first day after kidney transplantation

	AUC (95% CI)	*P*-value
Day 1 (8 to 12 h after KTx surgery)		
Single		
SCr (mg/dl)	0.732 (0.577 – 0.888)	0.012
△ $SCr_{day\ 1-\ day0}$ (%)	0.755 (0.603 – 0.907)	0.006
△ $SCr_{day\ 2-\ day1}$ (%)	0.783 (0.643 – 0.923)	0.002
uNGAL (ng/mL)	0.884 (0.773 – 0.996)	< 0.001
MDA (µmol/L)	0.900 (0.807 – 0.994)	< 0.001
Leptin (ng/mL)	0.759 (0.597 – 0.921)	0.008
CysC (mg/L)	0.914 (0.822 – 1.000)	< 0.001
Combined		
SCr + uNGAL	0.878 (0.763 – 0.992)	< 0.001
SCr + MDA	0.902 (0.811 – 0.992)	< 0.001
SCr + Leptin	0.792 (0.649 – 0.935)	0.003
SCr + CysC	0.914 (0.820 – 1.000)	< 0.001
SCr + CysC + MDA	0.960 (0.905 – 1.000)	< 0.001
SCr + CysC + uNGAL	0.918 (0.823 – 1.000)	< 0.001
SCr + CysC + MDA + uNGAL	0.939 (0.863 – 1.000)	< 0.001
SCr + CysC + MDA + uNGAL + Leptin	0.950 (0.882 – 1.000)	< 0.001

Abbreviations: SCr = serum creatinine; uNGAL = urinary neutrophil gelatinase-associated lipocalin; MDA = serum malondialdehyde; CysC = serum cystatin C; ln = logarithm
△$SCr_{day1-\ day0}$ and △$SCr_{day2-\ day1}$ = percentage changes in SCr between day 1 and day 0 and between day 2 and day 1.

Table 4. Results of multivariable logistic regression analysis of predictors of delayed graft function, after backward elimination of variables routinely associated to delayed graft function.

	OR	*P*-value	95% CI
SCr (per unit increase)	1.164	0.572	0.688 – 1.970
Cystatin C (per unit increase)	4.176	0.023	1.219 – 14.303
MDA (per 10 units increase)	5.360	0.048	1.016 – 28.276

Note: Results given by logistic regression. Recipient age, pre-transplant time on dialysis, HLA mismatches, and donor status (living/deceased) were not retained in the final model after backward elimination. Serum creatinine was forced to be included in the model, using the enter method.
Abbreviations: OR= odds ratio; 95% CI (95% confidence interval); SCr = serum creatinine; MDA = Serum malondialdehyde; CysC = Serum cystatin C.

Performance of the Triple-Biomarker Approach, including SCr, MDA, and CysC

For DGF (n=18) versus all non-DGF (n=22), the formula achieved by fitting the multiple logistic regression model for combining SCr, MDA, and CysC measured 8 to 12 hours after KTx was as follows: (−12.062 + 0.152 × SCr + 1.429 × CysC + 16.789 × MDA).

Using this formula and calculating the predicted values, the optimal sensitivity and specificity occurred at a value of 0.278 (sensitivity: 100%; specificity: 86%; positive and negative predictive value: 88% and 100%, respectively; and positive and negative likelihood ratios of 7.35 and 0, respectively).

DISCUSSION

We investigated the usefulness of combining SCr with four new biomarkers for predicting DGF after KTx. A triple-marker approach, using SCr, CysC, and MDA measured 8 to 12 h after KTx, was the most informative combination, resulting in an increased ability (AUC=0.96) to distinguish patients with graft damage and those who would require dialysis within the first week.

Delayed graft function burden is not only clinical but also financial due to the need for dialysis after transplant, additional laboratory and imaging studies, allograft biopsies, and longer hospital stays. In the long term, patients with DGF are 1.53 times more susceptible to graft loss at 5 years and have an overall 10% lower graft survival rate compared with patients with prompt graft function [1-3]. Because of the deleterious impact of DGF on perioperative care and graft outcomes, significant efforts have been undertaken to understand the pathogenesis of and the biological factors associated with this clinical condition. A variety of biological elements have been proposed for predicting DGF, but no suitable biomarkers are currently used routinely in clinical practice [9-11, 16, 17]

Allograft function following KTx is commonly monitored using SCr, even given the inaccuracy of this marker due to the impact of age, sex, and muscle mass on creatinine generation and its inability to detect functional impairment of less than 50% [8, 19]. These limitations hamper SCr from being a precise index of renal allograft function and compromise its value as a sensitive and reliable tool in the clinical management of KTx recipients. Therefore, interest has arisen in alternative markers of renal function, and CysC is an emerging endogenous marker of glomerular filtration rate. Cystatin C is a 13.3-kDa protein produced in all nucleated cells, which is released into the circulation at a constant rate and is not affected by other physiological or pathological changes and is then freely filtered by the glomeruli without tubular reabsorption or secretion. Cystatin C meets all of the criteria of a reliable marker of glomerular filtration rate, and several

studies have described the superiority of serum CysC over SCr for diagnosing filtration failure, both in native kidney disease patients [20-22] and in KTx recipients [23-25].

Although not a marker of glomerular filtration, NGAL, in both the urine and plasma, has been considered a promising biomarker of AKI and DGF in several settings [26-28]. NGAL is a 25-kDa protein normally expressed at low levels in multiple tissues, and it is excreted in the urine in small amounts. Nevertheless, after renal insult, both urine and plasma NGAL are increased, as occurs following ischemia-reperfusion injury in transplanted kidneys. Induction of NGAL after kidney injury precedes the elevation of classical markers of kidney damage. Given these data, levels of NGAL were shown to predict dialysis requirements within the first week, notably preceding the postoperative peak in SCr levels, which only occurred between the second and fourth days [9, 16, 27, 29].

Malondialdehyde was recently identified by our group as an early marker of graft dysfunction and a predictor of DGF [17]. Malondialdehyde is the most studied product of polyunsaturated fatty acid peroxidation [30, 31]; therefore, it has been frequently used as a biomarker of oxidative stress in several settings [32-36]. Oxidative stress is one of the most important components of the ischemia-reperfusion process [37-41], which is an inevitable phenomenon in KTx. Accordingly, we showed that elevated MDA levels reflected dysfunction of the kidney and predicted the need for renal replacement therapy within the first week following KTx better than SCr [17].

Along with MDA, leptin was another biomarker that was first hypothesized by our group to be an indicator of graft dysfunction. To date, most of the research on leptin has been focused on its associations with metabolic and cardiovascular health. So far, no reported studies have examined the clinical utility of leptin in the diagnosis of graft dysfunction after KTx. To the best of our knowledge, we addressed this question for the first time, and we confirmed that leptin levels slightly outperformed the traditional biomarker SCr in assessing the prognosis of DGF (ongoing study).

Our previous research concentrated on single biomarkers for DGF diagnosis. The present study focused on combining biomarkers to diagnose DGF more efficiently. Due to the complexity and overlapping pathophysiological mechanisms contributing to DGF and the distinct expression of biomarkers, it is unlikely that a single biomarker would be sufficient for accurate and reliable diagnosis of graft damage and for determining DGF prognosis. Consequently, the incorporation of several markers into a diagnostic or prognostic panel will likely be required to profile acute graft injury and its effects [15, 42]. Malondialdehyde and CysC evaluate different aspects and/or areas of kidney injury: MDA appears to be a more specific marker of ischemic-reperfusion, whereas CysC is a more functional marker of the glomerular filtration rate rather than of injury. Given these findings, combining these two biological pathways through these markers seems to increase their predictive value

beyond that of SCr in providing an early diagnosis of DGF. We examined different biomarker combinations, and the performance of these three combined markers was the best one achieved.

Although uNGAL has shown promising results as a predictor of DGF, the performance of the combinations that comprise uNGAL was lower than the triple-marker approach with SCr, MDA, and CysC. A possible explanation for the inferior performance of uNGAL might have been the lack of samples in some recipients with transient anuria. In our study, 5 recipients were anuric the morning following graft reperfusion, resulting in 12.5% of our patients not having urine samples to measure uNGAL at that particular time point. These patients could not be included in any of the approaches that included uNGAL, which reduced the sample size and the performance of uNGAL combinations. We chose to measure NGAL in urine instead of blood because uNGAL more accurately represents tubule damage in the kidney than filtration from the blood and because of the non-invasive nature of sample collection and the reduced number of interfering proteins. However, despite the unquestionable value of urinary markers, their use in transplant recipients could also be a drawback because of possible transient graft anuria and, consequently, the inability to obtain a urine sample.

This paper explored the optimal marker combination for DGF diagnosis using logistic regression. Combining multiple biomarkers for clinical use remains a challenge. The additional diagnostic and prognostic information gained by any biomarker over an established marker must be determined using adequate statistical tools. We focused particularly on improving the sensitivity and specificity in order to increase the accuracy of the diagnosis of acute graft dysfunction and of dialysis requirement prognosis. A very large number of biomarkers fail to be used in clinical practice due to the weakness of their clinical performance, namely, low sensitivity and low specificity [43]. After successful discovery and validation, some biomarkers fail in their ability to contribute decisively to patient care because they provide some incremental, but clinically not essential, information [44, 45]. The current study identified a simple and clinically applicable tool for detecting graft dysfunction earlier than SCr alone. For practical purposes, the concentrations of SCr, MDA, and CysC can be easily measured in routine blood samples, and the results can be replaced in the fitted formula. The final score would allow for the identification of kidneys with significant ischemia-reperfusion injury, which could result in easier clinical decision-making and more effective recipient management, thereby improving outcomes.

We previously described the strengths and limitations of this observational study. Given our relatively small sample size, larger studies should be conducted in the transplant setting to verify our findings. We designed a prospective, longitudinal study to identify

early markers of graft dysfunction during the KTx peritransplant period and to investigate their accuracy in predicting DGF. Our group first hypothesized two of the four biomarkers tested as early markers of kidney graft dysfunction. Given these hypotheses, we believed that it would be imprudent and costly to recruit a larger cohort, particularly because each patient was subjected to five measurements of each marker.

Despite these limitations, the current results suggested that the combined use of SCr, MDA, and CysC could be an important tool for the early determination of allograft function and thereafter for identifying individuals at the greatest risk of developing DGF. A major problem in selecting a biomarker profile is the proportional increase in economic burden. Thus, a “parsimonious” biomarker combination must be used in a cost-effective manner, and this consideration was an additional reason for choosing a combination of two, and not more, new markers to be added to the traditional marker.

In summary, combining biomarkers from different pathophysiologic pathways seems to be rational and a reliable strategy for optimizing sensitivity and specificity and obtaining additive diagnostic and prognostic information. In this study, we used multiple logistic regressions to combine several biomarkers, and a triple-marker approach, using SCr, MDA, and CysC, showed added value for the early detection of DGF.

FUNDING SOURCES

The authors recognize and thank Astellas Pharma Portugal for its valuable contributions in supporting the acquisition of reagents for measuring MDA and Abbott Laboratories for providing the kits for measuring urinary NGAL in almost 200 samples. The remaining NGAL kits were financed by the Multidisciplinary Unit for Biomedical Investigation, Porto, Portugal, which was funded by grants from the Foundation for Science and Technology (Fcomp-01-0124-FEDER-015896).

DISCLOSURE POLICY

The authors declare no conflicts of interest regarding the publication of this article.

The results presented in this paper have not been published previously, in whole or in part.

Role of the Sponsors: Astellas Pharma Portugal and Abbot Laboratories played no roles in the design or conducting of the study, the collection, management, analysis, or interpretation of the data, or the preparation or approval of the manuscript.

ACKNOWLEDGEMENTS

The investigators thank Dr. António Cabrita for permitting the study and the nursing and laboratory staff for their invaluable contributions to this work.

Author Contributions: Isabel Fonseca had full access to the data from the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Isabel Fonseca

Acquisition of the data and patient recruitment: Isabel Fonseca, Manuela Almeida, La Salete Martins, Leonídio Dias, Josefina Santos, Sofia Pedroso, and António Castro Henriques

Technical support and laboratory analysis: Henrique Reguengo and José Carlos Oliveira

Statistical analysis and interpretation of the data: Isabel Fonseca and Denisa Mendonça

Drafting of the manuscript: Isabel Fonseca

Critical revision of the manuscript for important intellectual content: Denisa Mendonça, Jorge Malheiro, and António Castro Henriques; all of the authors approved the final version

Study supervision: Denisa Mendonça, António Castro Henriques, and Luísa Lobato

REFERENCES

[1] Kyllonen LE, Salmela KT, Eklund BH, Halme LE, Hockerstedt KA, Isoniemi HM, et al. Long-term results of 1047 cadaveric kidney transplantations with special emphasis on initial graft function and rejection. Transpl Int. 2000;13:122-8.

[2] Ojo AO, Hanson JA, Wolfe RA, Leichtman AB, Agodoa LY, Port FK. Long-term survival in renal transplant recipients with graft function. Kidney Int. 2000;57:307-13.

[3] Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed graft function: risk factors and implications for renal allograft survival. Transplantation. 1997;63:968-74.

[4] Ponticelli C. Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. Nephrol Dial Transplant. 2014;29:1134-40.

[5] Rodrigo E, Minambres E, Ruiz JC, Ballesteros A, Pinera C, Quintanar J, et al. Prediction of delayed graft function by means of a novel web-based calculator: a single-center experience. Am J Transplant. 2012;12:240-4.

[6] Irish WD, McCollum DA, Tesi RJ, Owen AB, Brennan DC, Bailly JE, et al. Nomogram for predicting the likelihood of delayed graft function in adult cadaveric renal transplant recipients. J Am Soc Nephrol. 2003;14:2967-74.

[7] Machnicki G, Pinsky B, Takemoto S, Balshaw R, Salvalaggio PR, Buchanan PM, et al. Predictive ability of pretransplant comorbidities to predict long-term graft loss and death. Am J Transplant. 2009;9:494-505.

[8] Slocum JL, Heung M, Pennathur S. Marking renal injury: can we move beyond serum creatinine? Transl Res. 2012;159:277-89.

[9] Parikh CR, Jani A, Mishra J, Ma Q, Kelly C, Barasch J, et al. Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant. 2006;6:1639-45.

[10] Welberry Smith MP, Zougman A, Cairns DA, Wilson M, Wind T, Wood SL, et al. Serum aminoacylase-1 is a novel biomarker with potential prognostic utility for long-term outcome in patients with delayed graft function following renal transplantation. Kidney Int. 2013;84:1214-25.

[11] Zaza G, Rascio F, Pontrelli P, Granata S, Stifanelli P, Accetturo M, et al. Karyopherins: potential biological elements involved in the delayed graft function in renal transplant recipients. BMC Med Genomics. 2014;7:14.

[12] Pianta TJ, Peake PW, Pickering JW, Kelleher M, Buckley NA, Endre ZH. Clusterin in Kidney Transplantation: Novel Biomarkers Versus Serum Creatinine for Early Prediction of Delayed Graft Function. Transplantation. 2014.

[13] Cook NR. Statistical evaluation of prognostic versus diagnostic models: beyond the ROC curve. Clin Chem. 2008;54:17-23.

[14] Gastwirth JL. The statistical precision of medical testing procedures: applications to polygraph/AIDS antibodies test data. Statistical Science. 1987;2:213-38.

[15] Maiese K. Marking the onset of oxidative stress: biomarkers and novel strategies. Oxidative medicine and cellular longevity. 2009;2:1.

[16] Fonseca I, Oliveira JC, Almeida M, Cruz M, Malho A, Martins LS, et al. Neutrophil gelatinase-associated lipocalin in kidney transplantation is an early marker of graft dysfunction and is associated with one-year renal function. J Transplant. 2013;2013:650123.

[17] Fonseca I, Reguengo H, Almeida M, Dias L, Martins LS, Pedroso S, et al. Oxidative stress in kidney transplantation: malondialdehyde is an early predictive marker of graft dysfunction. Transplantation. 2014;97:1058-65.

[18] Grimes DA, Schulz KF. Refining clinical diagnosis with likelihood ratios. Lancet. 2005;365:1500-5.

[19] Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts. Clin Chem. 1992;38:1933-53.

[20] Dharnidharka VR, Kwon C, Stevens G. Serum cystatin C is superior to serum creatinine as a marker of kidney function: a meta-analysis. Am J Kidney Dis. 2002;40:221-6.

[21] Laterza OF, Price CP, Scott MG. Cystatin C: an improved estimator of glomerular filtration rate? Clin Chem. 2002;48:699-707.

[22] Roos JF, Doust J, Tett SE, Kirkpatrick CM. Diagnostic accuracy of cystatin C compared to serum creatinine for the estimation of renal dysfunction in adults and children--a meta-analysis. Clin Biochem. 2007;40:383-91.

[23] Poge U, Gerhardt T, Stoffel-Wagner B, Palmedo H, Klehr HU, Sauerbruch T, et al. Cystatin C-based calculation of glomerular filtration rate in kidney transplant recipients. Kidney Int. 2006;70:204-10.

[24] White C, Akbari A, Hussain N, Dinh L, Filler G, Lepage N, et al. Estimating glomerular filtration rate in kidney transplantation: a comparison between serum creatinine and cystatin C-based methods. J Am Soc Nephrol. 2005;16:3763-70.

[25] Maillard N, Mariat C, Bonneau C, Mehdi M, Thibaudin L, Laporte S, et al. Cystatin C-based equations in renal transplantation: moving toward a better glomerular filtration rate prediction? Transplantation. 2008;85:1855-8.

[26] Haase M, Bellomo R, Devarajan P, Schlattmann P, Haase-Fielitz A. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. Am J Kidney Dis. 2009;54:1012-24.

[27] Hollmen ME, Kyllonen LE, Inkinen KA, Lalla ML, Salmela KT. Urine neutrophil gelatinase-associated lipocalin is a marker of graft recovery after kidney transplantation. Kidney Int. 2011;79:89-98.

[28] Ronco C, Cruz D, Noland BW. Neutrophil gelatinase-associated lipocalin curve and neutrophil gelatinase-associated lipocalin extended-range assay: a new biomarker approach in the early diagnosis of acute kidney injury and cardio-renal syndrome. Seminars in nephrology. 2012;32:121-8.

[29] Bolignano D, Donato V, Coppolino G, Campo S, Buemi A, Lacquaniti A, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a marker of kidney damage. Am J Kidney Dis. 2008;52:595-605.

[30] Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med. 1990;9:515-40.

[31] Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative medicine and cellular longevity. 2014;2014:360438.

[32] Del Rio D, Stewart AJ, Pellegrini N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis. 2005;15:316-28.

[33] Pande D, Negi R, Karki K, Khanna S, Khanna RS, Khanna HD. Oxidative damage markers as possible discriminatory biomarkers in breast carcinoma. Transl Res. 2012;160:411-8.

[34] Ma MC, Chen YS, Huang HS. Erythrocyte oxidative stress in patients with calcium oxalate stones correlates with stone size and renal tubular damage. Urology. 2014;83:510 e9-17.

[35] Romero MJ, Bosch-Morell F, Romero B, Rodrigo JM, Serra MA, Romero FJ. Serum malondialdehyde: possible use for the clinical management of chronic hepatitis C patients. Free Radic Biol Med. 1998;25:993-7.

[36] Polidori MC, Savino K, Alunni G, Freddio M, Senin U, Sies H, et al. Plasma lipophilic antioxidants and malondialdehyde in congestive heart failure patients: relationship to disease severity. Free Radic Biol Med. 2002;32:148-52.

[37] Billings FTt, Ball SK, Roberts LJ, 2nd, Pretorius M. Postoperative acute kidney injury is associated with hemoglobinemia and an enhanced oxidative stress response. Free Radic Biol Med. 2011;50:1480-7.

[38] de Vries DK, Kortekaas KA, Tsikas D, Wijermars LG, van Noorden CJ, Suchy MT, et al. Oxidative damage in clinical ischemia/reperfusion injury: a reappraisal. Antioxid Redox Signal. 2013;19:535-45.

[39] Kim J, Seok YM, Jung KJ, Park KM. Reactive oxygen species/oxidative stress contributes to progression of kidney fibrosis following transient ischemic injury in mice. Am J Physiol Renal Physiol. 2009;297:F461-70.

[40] Waller HL, Harper SJ, Hosgood SA, Bagul A, Yang B, Kay MD, et al. Biomarkers of oxidative damage to predict ischaemia-reperfusion injury in an isolated organ perfusion model of the transplanted kidney. Free Radic Res. 2006;40:1218-25.

[41] Nastos C, Kalimeris K, Papoutsidakis N, Tasoulis MK, Lykoudis PM, Theodoraki K, et al. Global consequences of liver ischemia/reperfusion injury. Oxidative medicine and cellular longevity. 2014;2014:906965.

[42] Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. Am J Transplant. 2011;11:2279-96.

[43] Frangogiannis NG. Biomarkers: hopes and challenges in the path from discovery to clinical practice. Transl Res. 2012;159:197-204.

[44] Mas VR, Mueller TF, Archer KJ, Maluf DG. Identifying biomarkers as diagnostic tools in kidney transplantation. Expert Rev Mol Diagn. 2011;11:183-96.

[45] Roedder S, Vitalone M, Khatri P, Sarwal MM. Biomarkers in solid organ transplantation: establishing personalized transplantation medicine. Genome Med. 2011;3:37.

4.6. STUDY VI

THE IMPACT OF DELAYED GRAFT FUNCTION ON GRAFT AND PATIENT SURVIVAL USING A COMPETING EVENTS APPROACH

Fonseca I, Malheiro J, Teixeira L, Almeida M, Martins LS, Santos J, Dias L, Lobato L, Henriques AC, Mendonça D. The Effect of Delayed Graft Function on Graft and Patient Survival in Kidney Transplantation: An Approach using Competing Events Analysis. *Transplant International* 2015; 28: 738-50.

Delayed graft function is a common complication that affects kidney grafts immediately after transplantation. The impact of DGF in graft and patient survival is controversial. Some single-center studies have reported that DGF without rejection may have no impact on the long-term graft survival [273-275], but other investigators found that DGF is associated with a poor graft outcome independent of acute rejection [11, 32, 38]. There are also some controversies about the DGF impact on patient survival. This study reviewed our kidney transplant experience over the past three decades and focused on the impact of DGF with or without rejection on patient and long-term graft survival using a customary Cox survival analysis and a novel approach with competing events.

- Graft loss was defined as the absence of kidney function, occurring any time after transplantation due to either patient death with a functioning allograft ("patient death") or irreversible graft injury requiring chronic dialysis and/or retransplantation ("graft failure").

- Survival analysis was performed for evaluating graft and patient survival. First, estimates of cumulative incidence function (CIF) that accounts for competing risks were calculated and compared with the routinely used complement of Kaplan-Meier estimate (1-KM). The appropriate competing risks approach to estimate CIF resulted in a lower estimate of cumulative incidence. In other words, the actual probabilities of graft failure and patient death is wrongly overestimated using Kaplan-Meier method and the longer the duration of follow-up the larger the difference between the estimated by these two methods.

- The application of subdistribution regression model for competing risks showed that DGF by itself and independent of acute rejection has a detrimental effect on long-term graft survival, but not on patient survival.

TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

The effect of delayed graft function on graft and patient survival in kidney transplantation: an approach using competing events analysis

Isabel Fonseca,[1,2,3] Laetitia Teixeira,[4,3] Jorge Malheiro,[1,2] La Salete Martins,[1,2] Leonídio Dias,[1] António Castro Henriques[1,2] and Denisa Mendonça[4,3]

1 Department of Nephrology and Kidney Transplantation, Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal
2 Unit for Multidisciplinary Investigation in Biomedicine (UMIB), Porto, Portugal
3 EPIUnit-Institute of Public Health, University of Porto, Porto, Portugal
4 Department of Population Studies, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto, Porto, Portugal

Keywords
kidney transplantation, delayed graft function, competing risks analysis, long-term graft failure, patient death with graft function, subdistribution hazard regression model.

Correspondence
Isabel Fonseca, Department of Nephrology and Kidney Transplantation, Centro Hospitalar do Porto, Hospital de Santo António, Largo Prof. Abel Salazar, 4099-001 Porto, Portugal.
Tel.: +351914740001;
fax: 223 320 318
e-mail: isabelf27@gmail.com

Competing interests
The authors declare that they have no competing interests. The results presented in this study have not been published previously in whole or part, neither in abstract format.

Received: 19 October 2014
Revision requested: 30 November 2014
Accepted: 6 February 2015
Published online: 26 February 2015

doi:10.1111/tri.12543

Abstract

Objective: In kidney transplantation, the impact of delayed graft function (DGF) on long-term graft and patient survival is controversial. We examined the impact of DGF on graft and recipient survival by accounting for the possibility that death with graft function may act as a competing risk for allograft failure.
Study design and Setting: We used data from 1281 adult primary deceased-donor kidney recipients whose allografts functioned at least 1 year.
Results: The probability of graft loss occurrence is overestimated using the complement of Kaplan–Meier estimates (1-KM). Both the cause-specific Cox proportional hazard regression model (standard Cox) and the subdistribution hazard regression model proposed by Fine and Gray showed that DGF was associated with shorter time to graft failure (csHR = 2.0, $P = 0.002$; sHR = 1.57, $P = 0.009$), independent of acute rejection (AR) and after adjusting for traditional factors associated with graft failure. Regarding patient survival, DGF was a predictor of patient death using the cause-specific Cox model (csHR = 1.57, $P = 0.029$) but not using the subdistribution model.
Conclusions: The probability of graft loss from competing end points should not be reported with the 1-KM. Application of a regression model for subdistribution hazard showed that, independent of AR, DGF has a detrimental effect on long-term graft survival, but not on patient survival.

Introduction

Delayed graft function (DGF) is the most common complication affecting kidney allografts in the immediate post-transplant period. The rate of DGF after kidney transplantation (KTx) can vary from 2% to 50% depending on the definition and the practice center, and it is one of the most important risk factors for both acute rejection (AR) and impaired renal function at one year [1–4].

The impact of DGF on long-term graft survival is controversial [4]. Some single-center studies have reported limited or no impact of DGF on long-term graft survival in the absence of AR [5–8] while others have associated DGF with poor graft outcome independent of rejection [9–12]. Some authors have examined the association between DGF and patient survival, also with conflicting findings. Some of those studies reported no association between DGF with patient death with a functioning graft [6, 13], whereas

others showed a negative effect of DGF on survival of KTx recipients [9, 14–17].

Survival analysis is used to analyze time-to-event data and is commonly used in medical research [18]. In KTx, the Kaplan–Meier curves are one of the most used methodologies to study graft and patient survival, which censor all but one type of outcome. However, a patient can be at risk for more than one type of events and experience an event different from the outcome of interest. For example, when analyzing kidney allograft survival, the event of interest is chronic graft loss, but other events can be observed, namely patient death with graft function. These two events are termed competing risk events. That is, a competing risk is an event whose occurrence either precludes the occurrence of another event under examination or fundamentally alters the probability of occurrence of this other event [19, 20]. If a recipient dies, the decline and loss of graft function cannot be observed. Graft failure and patient death are competing end points that are mutually exclusive. Thus, appropriate methods accounting for the presence of competing risk events must be applied in the analysis and interpretation of such data.

Inappropriate methods such as the complement of Kaplan–Meier estimate (1-KM) have been applied to estimate probabilities of the occurrence of an event of interest in a competing risks setting [21] [19, 21–23]. This method produces biased estimates of end point probabilities because does not account for the various types of potential outcomes [20, 24]. In other words, the probability of an event of interest (e.g., graft failure) is estimated in an ideal world in which the other types of events do not exist (patient death, for example). Thus, when competing risks are present, cumulative incidence function (CIF) is the appropriate tool to analyse such data [22, 25]. Cumulative incidence function for a specific event, also known as the subdistribution function, is defined as the probability of failing from a given cause in the presence of competing events, given that a subject has survived or has already failed from different causes [20, 26, 27]. In other words, the cumulative incidence denotes the expected proportion of patients with a certain event over the course of time [22].

In the competing risks context and depending on the purpose of the study, there are different methods to quantify the effect of a covariate [22, 23]. The most common methods are the regression on cause-specific hazards using the competing risks analog to the Cox proportional hazards model, and the regression model for the cumulative incidence function proposed by Fine and Gray [26]. This method is based on the hazard of the subdistribution, providing a simple relationship between covariates and CIF, and is recommended for a competing risk approach [20, 26, 28]. As in any other regression analysis, modeling CIF can be used to identify potential prognostic factors for a particular failure in the presence of competing risks or to assess a prognostic factor of interest after adjusting for other potential risk factors in the model [27].

The kidney transplant program at our center began in 1983. From that time to the present, the rates of DGF varied due to the distinctive immunosuppressive protocols introduced, the inclusion of kidneys from living donors, and more recently the inclusion of expanded-criteria donors (ECD). We reviewed our KTx experience over the past three decades to study what effect evolving DGF (with and without AR associated) had on patient and long-term kidney transplant outcomes. Our analysis further supplements the current state of knowledge by assessing the impact of DGF on graft and recipient survival and by accounting for the possibility that death with graft function may act as a competing risk for allograft failure.

Materials and methods

Subjects and study design

This retrospective single-center study used data from the renal transplant database of the Department of Nephrology and Kidney Transplantation of Centro Hospitalar do Porto. Analyses were conducted on data from adult recipients who received a primary deceased-donor kidney transplant from August 1983 through December 2012 at this center and had a functioning renal allograft for at least 1 year. Exclusion criteria were (i) patients younger than 18 years old (n = 144), (ii) multi-organ transplant recipients, including kidney–pancreas (n = 169), (iii) retransplants (n = 163), (iv) recipients of living kidney donor (n = 150), and (v) recipients whose allografts functioned <1 year (n = 196). Patients with missing data on DGF or AR were also excluded from the analysis (n = 33, 2.5% of the final cohort). Because organ donation after circulatory death is not performed in our country, all donations occurred after brain death.

All patients were followed up from the time of transplant until death, graft failure or until December 31, 2013. The study was approved for the Institutional Review Board of Centro Hospitalar do Porto.

Definitions, variable categorization and main outcomes

The primary exposure of interest was the development of DGF after transplantation, with or without AR. In the current study, DGF was defined as the need for dialysis during the first week after transplantation. This definition was the same over the observation period of the three decades. Acute rejection was defined as either biopsy-proven rejection or antirejection treatment without biopsy. A variable "DGF-AR" was created with four categories: neither DGF nor AR; only DGF; only AR; DGF and AR. The cause of

kidney disease was categorized into three groups representing glomerular disease, diabetes, and all other diseases.

The study sample was divided into four cohorts based on the times in which immunosuppressive medications were introduced into clinical practice ("Transplant Era"): "Era" *1*, before 1990, the time of azathioprine and cyclosporine, no microemulsion; "Era" 2, between 1990 and 1995, the era of cyclosporine microemulsion; "*Era*" 3, between 1996 and 2000, marked by mycophenolate mofetil introduction and by the wide use of antithymocyte globulin; and "Era" 4, after 2000, the time of sirolimus availability and wide use of tacrolimus.

Time on dialysis prior to transplant was categorized as < and ≥5 years. Peak panel reactive antibody level (PRA-peak) was categorized into two categories according to the cutoff of 10%.

"Female-donor mismatch" was labeled when a male recipient received a kidney from a female donor. Patients were grouped as female donor to male recipient or all other combinations (female to female, male to male, or male to female).

The difference between donor and recipient age (recipient age subtracted from donor age) was divided into four groups, each representing approximately 25% of the patients according to quartiles (1stQ: <−15 years; 2ndQ: ≥−15 and ≤−4 years; 3rdQ: >−4 and ≤+6 years; 4thQ: >+6 years). Donors over the age of 60 or donors over the age of 50 with two of the following were classified as ECD: history of high blood pressure, serum creatinine ≥1.5 mg/dl, or death resulting from a stroke.

Graft loss was defined as the absence of kidney function occurring any time after transplantation due to either patient death with a functioning allograft ("patient death") or irreversible graft injury requiring chronic dialysis and/or retransplantation ("graft failure").

Statistical analyses

Descriptives of baseline characteristics that were identified by univariate survival analysis (unadjusted) or traditionally considered as potential confounders for graft loss were calculated, and the results are shown across DGF-AR groups (Table 1). The following potential confounders were examined in unadjusted and adjusted multivariable models: (i) recipient factors (age, cause of ESRD, PRA-peak, time on dialysis prior to transplant, HCV infection status); (ii) donor factors (ECD versus standard deceased-donor); and (iii) transplant factors (number of HLA mismatches, donor-age difference, "female-donor mismatch", and Transplant Era). Continuous variables are expressed as the mean and standard deviation (SD), and categorical variables are expressed as proportions.

Survival analysis was performed for analyzing graft and patient survival. To analyze graft survival, the event of interest was graft failure and the competing risk event was patient death with graft function. To analyze patient survival, the event of interest was patient death with graft function and the competing end point was graft failure. Patients without any of these outcomes were censored at the date of their last recorded visit or at the end of the study period (December 2013).

First, estimates of CIF taking competing risks into account were calculated and compared with the (1-km) estimates. Second, regression models taking competing risks into account were carried out to analyze the effect of covariates in the graft and in the patient survival. This analysis was performed considering two types of hazard: cause-specific hazard and subdistribution hazard. Proportional cause-specific hazard regression models were performed using the standard Cox cause-specific hazard regression model, censoring all patients without the event of interest. An alternative model proposed by Fine and Gray [26] was the approach used in the current study to model the subdistribution hazard.

An exploratory analysis was performed to examine the unadjusted effect of the traditional potential confounders by fitting univariable models. The cause-specific hazard ratio (csHR) and the subdistribution hazard ratio (sHR) for graft loss either due to declining function or to patient death according to the primary exposure of interest (DGF-AR) were estimated in a multivariable analysis adjusting for the influence of these potential confounders. The group of categorical variables with lower proportion of the end point (graft failure or patient death) was considered as the reference class. Therefore, the 1st and the 4th quartiles of donor-age difference were considered the reference classes in graft and patient survival, respectively.

As the main objective of this study was to assess the prognostic value of a specific variable of interest (DGF-AR), we opted to study the impact of DGF-AR in graft and patient survival after adjusting for other risk factors traditionally considered as potential confounders in the model, even those that were nonsignificant. The impact of DGF-AR on graft and patient survival was similar when including in the model only the statistical significant variables (supplemental data).

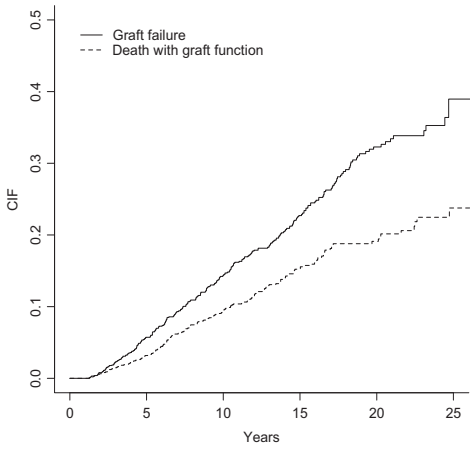
About 37.4% (n = 479) of patients had at least one variable missing. The main variable of interest DGF-AR and the survival outcome (patient death and graft failure) presented no missing values. Missing data were considered to be missing completely at random. Therefore, missing data were dealt by carrying complete case analyses, in which patients were excluded in multivariable analyses if the required variables were missing.

Statistical analyses were performed using SPSS version 22.0 (IBM SPSS Statistics, IBM Corporation, Chicago,

Table 1. Patient demographic and clinical characteristics by DGF-AR occurrence (n = 1281).

Characteristic	No DGF nor AR (n = 721)	DGF only (n = 274)	AR only (n = 175)	DGF + AR (n = 111)
Recipient				
Age (yr), mean (SD)	43.8 (12.3)	46.0 (12.3)	36.8 (12.2)	39.8 (12.4)
Gender				
Male	427 (54.9)	175 (22.5)	111 (14.3)	65 (8.4)
Female	294 (58.4)	99 (19.7)	64 (12.7)	46 (9.1)
Cause of ESRD (n, %)				
Glomerulonephritis	274 (57.7)	96 (20.2)	69 (14.5)	36 (7.6)
Diabetes	40 (56.3)	18 (25.4)	5 (7.0)	8 (11.3)
Other	407 (55.4)	160 (21.8)	101 (13.7)	67 (9.1)
Peak PRA (n, %)				
<10	533 (60.4)	1784 (20.8)	99 (11.2)	67 (7.6)
≥10	92 (45.8)	50 (24.9)	34 (16.9)	25 (12.4)
Unknown/missing	96 (48.7)	40 (20.3)	42 (21.3)	19 (9.6)
Time on dialysis (mo)				
Mean (SD)	3.9 (3.6)	4.2 (3.4)	3.3 (3.1)	4.0 (3.5)
≥ 5 years (n, %)	198 (57.6)	78 (22.7)	35 (10.2)	33 (9.6)
Unknown/missing	31 (57.4)	17 (31.5)	4 (7.4)	2 (3.7)
HCV infection (n, %)				
HCV-negative	608 (56.6)	229 (21.3)	151 (14.1)	86 (8.0)
HCV-positive	58 (48.3)	36 (30.0)	12 (10.0)	14 (11.7)
Unknown/missing	55 (63.2)	9 (10.3)	12 (13.8)	11 (12.6)
Donor				
Age (yr), mean (SD)	37.7 (14.5)	39.9 (14.9)	36.0 (12.9)	37.3 (12.4)
ECD (n, %)	84 (49.4)	55 (32.4)	18 (10.6)	13 (7.6)
Unknown/missing	9 (69.2)	2 (15.4)	1 (7.7)	1 (7.7)
Donor-Recipient				
Cold ischemia time (h)				
Mean (SD)	22.2 (8.6)	23.9 (4.9)	22.8 (4.1)	24.0 (4.6)
Unknown/missing (n, %)	389 (65.2)	107 (17.9)	63 (10.6)	38 (6.4)
HLA mismatches				
A	1.23 (0.67)	1.17 (0.70)	1.22 (0.63)	1.22 (0.61)
Unknown/missing	42 (64.6)	11 (16.9)	11 (16.9)	1 (1.5)
B	1.21 (0.68)	1.24 (0.67)	1.35 (0.67)	1.29 (0.71)
Unknown/missing	39 (61.9)	12 (19.0)	11 (17.4)	1 (1.6)
DR	0.68 (0.69)	0.61 (0.66)	0.74 (0.73)	0.63 (0.64)
Unknown/missing	35 (54.7)	13 (20.3)	12 (18.8)	4 (6.3)
Female-donor mismatch				
Yes	82 (33.2)	50 (20.2)	21 (8.5)	94 (38.1)
No	523 (61.4)	186 (21.8)	133 (15.6)	10 (1.2)
Unknown/missing	116 (63.7)	38 (20.9)	21 (11.5)	7 (3.8)
Donor-recipient age difference (n, %)				
≤ −15 yr than recipient	183 (61.4)	67 (22.5)	31 (10.4)	17 (5.7)
−15.1 to −4 yr than recipient	162 (54.4)	69 (23.2)	39 (13.1)	28 (9.4)
−4.1 to +6 yr than recipient	157 (52.7)	59 (19.8)	49 (16.4)	33 (11.1)
> + 6 yr than recipient	150 (52.1)	57 (19.8)	50 (17.4)	31 (10.8)
Unknown/missing (n, %)	69 (77.5)	12 (13.5)	6 (6.7)	2 (2.2)
Transplantation Era				
1983–1990	46 (29.5)	31 (19.9)	39 (25.0)	40 (25.6)
1990–1995	152 (49.4)	70 (22.7)	58 (18.8)	28 (9.1)
1996–2000	150 (55.8)	62 (23.0)	41 (15.2)	16 (5.9)
2001–2012	371 (69.2)	104 (19.4)	37 (6.9)	24 (4.5)

Percentages are calculated within DGF-AR status. ERSD, end-stage renal disease; ECD, expanded-criteria donors; HCV, Hepatitis C virus; SD, standard deviation; yr, year. No missing values for the variables: recipient age and gender, cause of ERSD, and transplantation era.



Number at risk						
Graft failure	1281	1000	722	506	301	191
Patient death	1281	1029	773	575	393	284

Figure 1 Cumulative incidence curves for all possible outcomes taking competing risks into account. CIF, Cumulative incidence function.

IL, USA) and R software using the packages *coxph* and *cmprsk*. A significance level of 0.05 was considered.

Results

Sample

The final sample included 1281 primary adult kidney recipients transplanted between 1983 and 2012. About 60.7% were male, and the overall mean age was 43.0 years (SD = 12.6). Median follow-up was 9.8 years (range 1.0–30.2 years). A total of 424 (33.1%) grafts were lost during the study period, either as a result of loss of function (n = 258, 60.8%) or patient death (n = 166, 39.2%). The main causes of patient death with graft function were cardiovascular disease (n = 63, 38.0%), followed by malignancies (n = 35, 21.1%) and infection (n = 26, 15.7%).

Cumulative incidence function

Figure 1 summarizes the cumulative incidence estimates for the two possible outcomes taking competing risks into accounts (the survival plots were halted at 25 years because the proportion of patients free of an event, but still in follow-up, becomes small). The probabilities of experiencing graft failure by 5, 10, and 20 years after KTx were 0.06, 0.14, and 0.32, respectively. The probabilities of death with graft function were 0.03, 0.09, and 0.19, respectively.

Cumulative incidence estimates versus the complement of Kaplan–Meier estimates

Figure 2 presents the curves for the CIF of the occurrence of the event of interest obtained using two different methods: taking competing risks into account and the 1-KM.

The appropriate competing risks approach to estimate CIF results in a lower estimate of cumulative incidence. The magnitude of the difference in the incidence of graft

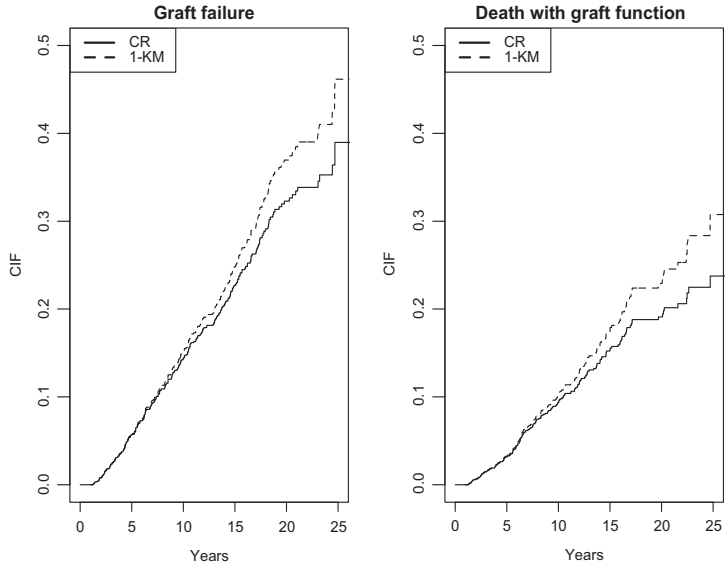


Figure 2 The complement of the Kaplan–Meier estimate and the cumulative incidence estimate for graft failure and death with graft function. CR, competing risks; 1-KM, Complement of Kaplan–Meier estimate.

failure and patient death, as calculated using the two methods, increases with the period of follow-up, mainly after the tenth year. In other words, the actual probabilities of graft failure and patient death are overestimated using the Kaplan–Meier method. Furthermore, the longer the duration of follow-up is the larger the difference between the estimates by these two methods.

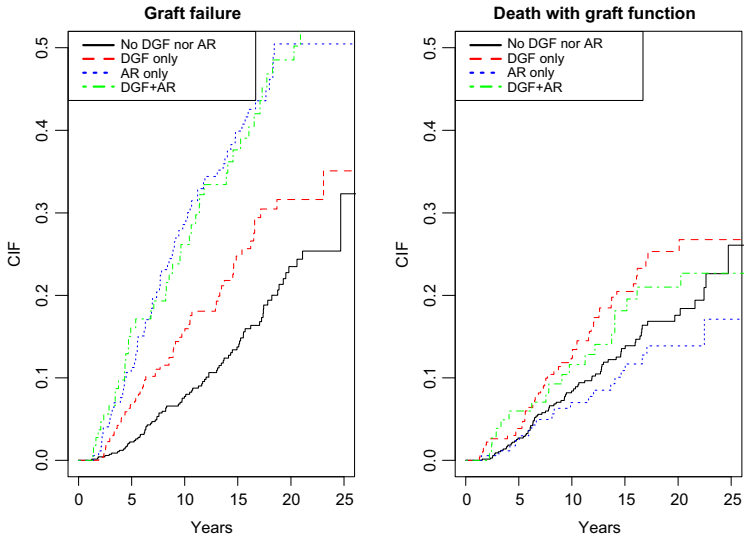
Delayed graft function and acute rejection

The overall incidence of DGF was 30.1% (385 grafts) and was not associated with AR in 274 grafts (21.4%) and was associated with AR in 111 (8.7%). The overall occurrence of DGF declined over decades from 45.5% in Era 1 to 31.8% in Era 2, 29.0% in Era 3, and 23.9% in Era 4. The overall incidence of AR similarly decreased from 50.6% in Era 1 to 27.9% in Era 2, 21.2% in Era 3, 11.4% in Era 4. The characteristics of the recipients according to DGF-AR status are summarized in Table 1.

The Fig. 3 displays the cumulative incidence curves for graft failure and death with graft function according to DGF-AR status. Differences were found between DGF-AR status with regard to the graft failure: all three categories of the variable DGF-AR (DGF only, AR only, and both DGF and AR) had a higher probability of graft failure than the non-DGF/non-AR category. Concerning patient survival, the differences between DGF-AR groups were not so pronounced.

The impact of DGF on graft and patient survival by Cox and Fine and Gray regression models

Tables 2 and 3 give a summary of the unadjusted and adjusted effects of covariates for graft failure and patient death with graft function based on the two types of models: the cause-specific hazard model (standard Cox proportional hazards regression) and the subdistribution hazard model (Fine and Gray model).



Number at risk at: (years)	0	5	10	15	20	25
Graft failure						
No DGF nor AR	721	555	392	268	148	88
DGF	274	221	166	115	78	55
AR	175	144	101	76	39	25
DGF + AR	111	80	63	47	36	23
Patient Death						
No DGF nor AR	721	552	388	267	157	96
DGF	274	228	173	123	88	65
AR	175	159	135	120	89	74
DGF + AR	111	90	77	65	59	49

Figure 3 Cumulative incidence curves for graft failure and death with graft function according to DGF-AR status. DGF, Delayed graft function; AR, acute rejection.

Table 2. Cox proportional hazard regression (cause-specific hazard model) for all possible events.

		Unadjusted models			Adjusted model (n = 802)		
		csHR	95% CI	*P* value	csHR	95% CI	*P* value
Graft Failure (censored for patient death)	**DGF-AR** (reference: non-DGF and non-AR)						
	Only DGF	1.79	1.28–2.48	0.001	2.00	1.30–3.07	0.002
	Only AR	2.99	2.17–4.10	<0.001	2.81	1.81–4.38	<0.001
	DGF + AR	3.37	2.35–4.84	<0.001	2.60	1.58–4.27	<0.001
	Transplant Era (reference: Era 4 > 2000)						
	Era 1 (<1990)	2.58	1.65–4.03	<0.001	3.21	1.47–7.04	0.004
	Era 2 (1990–1995)	2.03	0.34–3.09	0.001	3.64	1.73–7.62	0.001
	Era 3 (1996–2000)	0.92	0.57–1.49	0.74	1.66	0.78–3.55	0.19
	ECD (yes vs. no)	2.41	1.73–3.34	<0.001	2.49	1.35–4.60	0.003
	Donor-recipient age difference (reference: 1st Q: < - 15 yr)						
	2nd Q (≥−15 and ≤ −4 yr)	1.76	1.16–2.69	0.009	1.60	0.89–2.88	0.12
	3rd Q (> - 4 and ≤ +6 yr)	2.35	1.56–3.53	<0.001	1.73	0.94–3.21	0.081
	4th Q (> + 6 yr)	3.91	2.66–5.77	<0.001	2.62	1.32–5.20	0.006
	Cause of ESRD (reference: others)						
	Diabetic nephropathy	1.71	1.01–2.89	0.046	1.21	0.47–3.12	0.069
	Glomerulonephritis	1.12	0.87–1.45	0.38	0.75	0.75–1.47	0.77
	Time on dialysis (>5 vs. <5 yr)	1.24	0.93–1.65	0.14	1.42	0.97–2.06	0.13
	Peak PRA (>10 vs. <10)	1.26	0.92–1.73	0.15	0.93	0.63–1.35	0.69
	HCV infection (positive vs. negative)	1.43	1.02–2.00	0.039	1.12	0.73–1.70	0.61
	Female-donor mismatch (yes vs. no)	0.98	0.68–1.44	0.98	0.84	0.50–1.41	0.51
	HLA MM A (reference: 0)						
	1 vs. 0	1.18	0.80–1.74	0.19	1.19	0.71–1.97	0.51
	2 vs. 0	1.24	0.82–1.89	0.17	1.21	0.70–2.11	0.50
	HLA MM B (reference: 0)						
	1 vs. 0	1.04	0.71–1.52	0.77	1.16	0.71–1.92	0.55
	2 vs. 0	1.12	0.75–1.67	0.57	1.46	0.87–2.47	0.16
	HLA MM DR (reference: 0)						
	1 vs. 0	1.02	0.79–1.33	0.75	1.00	0.72–1.41	0.97
	2 vs. 0	0.65	0.36–1.15	0.11	0.74	0.34–1.59	0.44
	Recipient age (1-yr increase)	0.97	0.96–0.98	<0.001	0.99	0.97–1.01	0.27

Graft survival

The variables identified as significant predictors of graft failure in unadjusted cause-specific hazard models, and unadjusted Fine and Gray models were similar (Tables 2 and 3). In both statistical approaches, all three categories of the variable DGF-AR (DGF only, AR only, and both DGF and AR) had a deleterious effect on graft survival compared to the neither DGF nor AR category. The other covariates associated with graft failure were the Transplant Eras (Eras 1 and 2 vs. 4), grafts from ECD, donor-recipient age difference (2nd, 3rd, and 4th quartiles vs. 1st quartile), cause of ERSD (diabetes vs. other), positive HCV status (nearly reaching the significance level in Fine and Gray model) and recipient age.

In multivariable models (Tables 2 and 3, cause-specific and subdistribution hazard models, respectively), DGF with or without AR as well as AR only remained consistently associated with graft failure. Furthermore, recipients whose cause of ERSD was diabetes (nearly reaching the significance level in the cause-specific hazard model), transplantation in Eras 1 and 2, kidneys from ECD or from donors with an age difference of more than 6 years, were also associated with poor graft survival. In both models, when the donor-recipient age difference was added, the recipient's age became nonsignificant.

Patient survival

In relation to patient death with graft function, in the unadjusted cause-specific hazard models and unadjusted Fine and Gray models, the predictors of patient survival were slightly different among models (Tables 2 and 3). In the unadjusted cause-specific hazard models, the variables DGF (isolated or associated to AR), Transplant Era, ECD, donor-recipient age difference, cause of ERSD, pretransplant time on dialysis, PRA-peak, HCV status, and recipient age were significantly associated with patient death (Table 3). In the unadjusted Fine and Gray model, however, the variables identified as predictors of patient

Table 2. continued

		Unadjusted models			Adjusted model (n = 802)		
		csHR	95% CI	*P* value	csHR	95% CI	*P* value
Patient Death (censored for graft failure)	**DGF-AR** (reference: non-DGF and non-AR)						
	Only DGF	1.95	1.41–2.68	<0.001	1.57	1.05–2.35	0.029
	Only AR	1.29	0.85–1.95	0.23	1.19	0.69–2.03	0.54
	DGF + AR	2.36	1.57–3.54	<0.001	1.34	0.76–2.34	0.31
	Transplant Era (reference: Era 4 > 2000)						
	Era 1 (< 1990)	2.63	1.69–4.08	<0.001	5.29	2.59–10.8	<0.001
	Era 2 (1990–1995)	1.39	0.90–2.14	0.139	3.11	1.58–6.12	0.001
	Era 3 (1996–2000)	0.73	0.45–1.20	0.214	1.12	0.56–2.23	0.76
	ECD (yes vs. no)	1.58	1.05–2.37	0.027	0.82	0.39–1.70	0.59
	Donor-recipient age difference (reference: 4th Q: > +6 yr)						
	1st Q (<−15 yr)	1.54	1.04–2.28	0.03	0.55	0.28–1.11	0.55
	2rd Q (≥−15 and ≤ −4 yr)	1.10	0.72–1.68	0.65	0.70	0.38–1.31	0.70
	3rd Q (> - 4 and ≤ +6 yr)	1.14	0.74–1.74	0.56	0.79	0.45–1.38	0.79
	Cause of ESRD (reference: others)						
	Diabetic nephropathy	2.56	1.60–4.11	<0.001	4.07	2.03–8.18	<0.001
	Glomerulonephritis	0.76	0.57–1.02	0.071	0.90	0.62–1.32	0.60
	Time on dialysis (>5 vs. <5 yr)	1.71	1.28–2.29	<0.001	1.49	1.01–2.20	0.048
	Peak PRA (>10 vs. <10)	1.46	1.06–2.01	0.021	1.29	0.89–1.86	0.18
	HCV infection (positive vs. negative)	1.87	1.32–2.65	<0.001	1.56	1.00–2.41	0.048
	Female-donor mismatch (yes vs. no)	1.06	0.72–1.58	0.76	1.27	0.80–2.04	0.31
	HLA MM A (reference: 0)						
	1 vs. 0	0.75	0.51–1.10	0.15	0.86	0.53–1.39	0.53
	2 vs. 0	0.83	0.55–1.25	0.32	1.17	0.69–1.98	0.56
	HLA MM B (reference: 0)						
	1 vs. 0	1.27	0.82–1.98	0.47	1.19	0.69–2.03	0.54
	2 vs. 0	1.37	0.86–2.17	0.58	1.40	0.81–2.41	0.23
	HLA MM DR (reference: 0)						
	1 vs. 0	1.08	0.81–1.45	0.67	0.95	0.66–1.37	0.78
	2 vs. 0	1.22	0.74–2.00	0.07	1.18	0.65–2.14	0.59
	Recipient age (1-yr increase)	1.04	1.02–1.05	<0.001	1.05	1.03–1.08	<0.001

csHR, cause-specific hazard ratio; ERSD, end-stage renal disease; ECD, expanded-criteria donors; HCV, Hepatitis C virus; PRA, panel reactive antibody; HLA MM, HLA mismatches; yr, year; Q, quartile. The bold printed covariables indicate statistical significance in the multivariable model.

survival were as follows: DGF (DGF only vs. non-DGF/non-AR), Transplant Eras (Eras 1 and 2 vs. Era 4), donor-recipient age difference (1st, 2nd, and 3rd quartiles vs. 4th quartile), cause of ERSD (glomerulonephritis, associated with decreased HR), time on dialysis, PRA-peak, and recipient age.

Considering the multivariable regression models, the cause-specific hazard model showed that DGF only, Transplant Eras 1 and 2, diabetes as the cause of ESRD, pretransplant time on dialysis ≥5 years, positive HCV status and increasing recipient age had a deleterious effect on patient survival (Table 3). In the Fine and Gray adjusted model, only recipient age and Transplant Eras 1 and 2 were significantly associated with patient death (Table 4). In both models, Transplant Era (Era 2 vs. Era 4) emerged as significant when adjusted for any of the other variables included.

Unlike the adjusted cause-specific hazard model, the adjusted Fine and Gray model found that DGF was not significantly associated with patient death (csHR = 1.57, 95% CI = 1.05–2.35, $P = 0.029$ vs. sHR = 1.22, 95% CI = 0.85–1.76, $P = 0.28$). These differences are related to the different composition of the risk sets (in contrast to the cause-specific model where the DGF recipients who lost their graft were censored and removed from the risk set, in the subdistribution model, these same patients are maintained in the risk set) and to the increased risk of graft failure found for the recipients with DGF (these recipients had a 57% higher hazard risk of graft failure compared to non-DGF/non-AR: sHR = 1.57 95% CI = 1.12–2.21, $P = 0.009$).

Discussion

In this study, application of a regression model for subdistribution hazard showed that DGF, alone and independent of AR, has a significant detrimental effect on long-term graft survival but not on patient survival. Despite the

Table 3. Fine and Gray model (hazard of the subdistribution model) for all possible events.

		Unadjusted models			Adjusted model (n = 802)		
		sHR	95% CI	*P* value	sHR	95% CI	*P* value
Graft Failure	**DGF-AR** (reference: non-DGF and non-AR)						
	Only DGF	1.69	1.21–2.35	0.002	1.57	1.12–2.21	0.009
	Only AR	3.09	2.24–4.27	<0.001	2.57	1.85–3.56	<0.001
	DGF + AR	3.26	2.28–4.67	<0.001	2.26	1.52–3.37	<0.001
	Transplant Era (reference: Era 4 > 2000)						
	Era 1 (< 1990)	2.69	1.74–4.18	<0.001	1.87	1.11–3.14	0.019
	Era 2 (1990–1995)	2.23	1.49–3.33	0.001	1.93	1.23–3.03	0.004
	Era 3 (1996–2000)	1.02	0.64–1.65	0.93	0.96	0.58–1.60	0.88
	ECD (yes vs. no)	2.10	1.51–2.92	<0.001	1.73	1.09–2.74	0.019
	Donor-recipient age difference (reference: 1st Q: < −15 yr)						
	2nd Q (≥−15 and ≤ −4 yr)	1.82	1.20–2.76	0.005	1.56	0.97–2.50	0.066
	3rd Q (>−4 and ≤ +6 yr)	2.36	1.60–3.50	<0.001	1.65	1.01–2.70	0.049
	4th Q (> + 6 yr)	4.06	2.79–5.90	<0.001	2.52	1.44–4.41	0.001
	Cause of ESRD (reference: others)						
	Diabetic nephropathy	1.71	1.01–2.89	0.046	2.23	1.27–3.92	0.005
	Glomerulonephritis	1.12	0.87–1.45	0.38	1.08	0.83–1.41	0.57
	Time on dialysis (≥5 vs. <5 yr)	1.13	0.86–1.49	0.39	1.27	0.93–1.73	0.13
	Peak PRA (>10 vs. <10)	1.16	0.86–1.56	0.34	1.00	0.70–1.42	0.99
	HCV infection (positive vs. negative)	1.38	0.99–1.91	0.058	1.27	0.88–1.84	0.21
	Female-donor mismatch (yes vs. no)	0.96	0.66–1.42	0.85	1.02	0.69–1.49	0.94
	HLA MM A (reference: 0)						
	1 vs. 0	1.29	0.89–1.87	0.19	1.00	0.69–1.45	1.00
	2 vs. 0	1.33	0.89–1.99	0.17	1.12	0.75–1.68	0.57
	HLA MM B (reference: 0)						
	1 vs. 0	1.05	0.74–1.51	0.77	1.08	0.73–1.59	0.71
	2 vs. 0	1.12	0.76–1.63	0.57	1.31	0.88–1.96	0.19
	HLA MM DR (reference: 0)						
	1 vs. 0	1.04	0.81–1.35	0.75	1.08	0.82–1.43	0.57
	2 vs. 0	0.64	0.37–1.11	0.11	0.80	0.47–1.37	0.42
	Recipient age (1-yr increase)	0.97	0.96–0.98	<0.001	0.99	0.97–1.01	0.20

common use in clinical cancer research, the estimation of CIF and the application of competing risks models in nephrology is relatively recent [23, 29–38]. To the best of our knowledge, this is the first study that used a competing risks approach to address the impact of DGF on graft and patient survival.

Some previous studies have suggested that DGF without AR may have no impact on long-term graft survival [5–8]. Consistent with other reports [9–11], using both of the statistical approaches, our findings support that DGF per se is an independent predictor of graft failure. In fact, after adjusting for most of the factors traditionally associated with graft failure, early kidney dysfunction has a clear adverse effect on long-term graft survival meaning that the presence or absence of DGF will give an indication of the life expectancy of the kidney graft.

In addition to the DGF-AR status, the other factors independently associated with graft failure were, as expected, Transplant Eras 1 and 2, grafts from ECD donors, diabetes as a cause of ERSD and increasing donor-recipient age difference. Compared to donors who were more than 15 years younger than their recipients, all other categories showed a trend toward an increased risk of graft failure, including the category of donors who were 4–15 years younger than the recipient, with a near significant hazard of failure by the subdistribution approach. This finding was somewhat unexpected. The donor-recipient age difference was studied mostly in recipients from living donors. Grafts donated by live donors who were significantly older than recipients had similar graft and patient survival compared to recipients who received organs of a similar vintage [29, 39]. Shin *et al.* [40] evaluated whether the effect of donor age was different according to recipient age (≤−21, −20 to −1, 0–20, and ≥ 21 years) in kidneys from deceased donors. The authors confirmed that a negative donor-recipient age difference (recipients receiving kidneys from a donor younger than the recipient) was associated with greater death-censored graft survival. Our findings are in the line with this study. However, we did not expect that the narrow difference of donor-recipient age that we

Table 3. continued

		Unadjusted models			Adjusted model (n = 802)		
		sHR	95% CI	*P* value	sHR	95% CI	*P* value
Patient	DGF-AR (reference: non-DGF and non-AR)						
Death	Only DGF	1.53	1.08–2.19	0.018	1.22	0.85–1.76	0.28
	Only AR	0.80	0.49–1.30	0.370	0.84	0.50–1.41	0.51
	DGF + AR	1.29	0.78–2.15	0.320	1.10	0.63–1.93	0.74
	Transplant Era (reference: Era 4 > 2000)						
	Era 1 (< 1990)	1.80	1.09–2.95	0.021	3.74	2.00–7.02	<0.001
	Era 2 (1990–1995)	1.29	0.81–2.04	0.280	2.13	1.25–3.62	0.005
	Era 3 (1996–2000)	0.75	0.44–1.27	0.280	1.03	0.59–1.78	0.93
	ECD (yes vs. no)	1.14	0.70–1.85	0.60	0.88	0.49–1.71	0.70
	Donor-recipient age difference (reference: 4th Q: > +6 yr)						
	1st Q (<−15 yr)	3.07	1.90–4.95	<0.001	1.20	0.65–2.20	0.57
	2rd Q (≥−15 and ≤ −4 yr)	1.71	1.01–2.88	0.044	1.19	0.68–2.08	0.56
	3rd Q (>−4 and ≤ +6 yr)	1.82	1.07–3.10	0.027	1.54	0.90–2.65	0.12
	Cause of ESRD (reference: others)						
	Diabetic nephropathy	1.41	0.75–2.64	0.28	1.84	0.97–3.49	0.064
	Glomerulonephritis	0.62	0.44–0.88	0.007	0.76	0.54–1.07	0.12
	Time on dialysis (≥ 5 vs. <5 yr)	1.62	1.17–2.24	0.004	1.24	0.86–1.78	0.25
	Peak PRA (>10 vs. <10)	1.61	1.14–2.27	0.007	1.29	0.89–1.86	0.18
	HCV infection (positive vs. negative)	1.39	0.92–2.11	0.12	0.99	0.62–1.57	0.95
	Female-donor mismatch (yes vs. no)	1.21	0.80–1.85	0.37	1.37	0.91–2.07	0.13
	HLA MM A (reference: 0)						
	1 vs. 0	0.74	0.49–1.11	0.15	0.79	0.52–1.19	0.26
	2 vs. 0	0.80	0.51–1.25	0.32	0.84	0.53–1.35	0.47
	HLA MM B (reference: 0)						
	1 vs. 0	1.18	0.75–1.86	0.47	1.09	0.70–1.71	0.70
	2 vs. 0	1.14	0.71–1.83	0.58	1.00	0.62–1.62	0.99
	HLA MM DR (reference: 0)						
	1 vs. 0	1.08	0.77–1.5	0.67	1.00	0.72–1.41	0.98
	2 vs. 0	1.62	0.97–2.71	0.07	1.43	0.85–2.42	0.18
	Recipient age (1-yr increase)	1.05	1.04–1.06	<0.001	1.06	1.04–1.08	<0.001

sHR, subdistribution hazard ratio; ERSD, end-stage renal disease; ECD, expanded-criteria donors; HCV, Hepatitis C virus; PRA, panel reactive antibody; HLA MM, HLA mismatches; yr, year; Q, quartile. The bold printed covariables indicate statistical significance in the multivariable model.

considered would have a significant effect. We believe that this result emphasizes the advantage of young donors for long-term graft survival.

No clear effect of DGF on patient outcome has been reported. Some studies highlight the association between DGF and mortality, [9, 14, 17] whereas others [6, 8, 13] have not found a significant effect. None of these studies accounted for competing risks.

In our study, we confirmed this association using a standard Cox proportional hazards regression, but not when modeling cumulative incidence of the failure types (Fine and Gray models). Both approaches are valid, and the choice of the appropriate approach depends on the research question. To better understand and discuss this finding, we first give an overview of competing risks in the context of KTx.

Survival analysis involves the statistical analysis of the time to the occurrence of an event. However, in biomedical research, the need to address multiple potential outcomes is nearly ubiquitous. Competing risks are used to model a situation in which subjects under investigation are exposed to several causes of failure, such as graft failure or death with graft function. These two events are mutually exclusive, and only the first event that occurs is observed. Thus, the analysis and interpretation of competing risk data differ from survival analysis with only a single cause of failure. As such appropriate methods must be applied.

The estimated cumulative incidence of an event of interest using the 1-KM estimate is, in general, higher than estimates obtained when accounting for competing risks [19, 41, 42]. This is because when an individual experiences a competing risk event, this individual is treated as censored and is eliminated from the risk set. Censored patients are considered to have the same probability of experiencing the event as patients who remain under follow-up [41]. However, a subject who is censored due to failure from a competing risk (e.g., patient death) will clearly not experience the event of interest (allograft loss functioning). Because subjects who will never fail (by the failure of interest) are

treated as if they could fail (they are censored), the 1-km estimator overestimates the probability of failure and underestimates the corresponding survival probability [19, 42]. We confirmed this finding, especially after the 10th year of follow-up when the probability of graft failure and patient death increases.

In the competing risk context, there are different approaches to quantifying the effect of covariates in the presence of competing events [26, 43]. In the current study, the influence of DGF was evaluated using the cause-specific Cox proportional hazards regression model (modeling the cause-specific hazard) and the Fine and Gray regression model (modeling the subdistribution hazards). We found that the effect of this covariate differed between these two approaches. Both results are valid, but their interpretation is different and depends on the purpose of the study (etiology vs. prediction) [23, 42, 44, 45].

If the primary interest in the etiological question of how the covariates affect the event of interest, the cause-specific hazards model would be most appropriate, because they directly model the covariate effect on event rates among subjects at risk [28]. Using this approach in the current study, DGF significantly increases the risk of mortality (csHR = 1.57, $P = 0.029$). This hazard can be interpreted among those recipients who did not experience the event of interest (patient death), that is, those recipients who were censored because they were alive or had already been transferred for dialysis due to graft failure (competing event), but they were alive when they were censored for graft failure. Considering our example, the csHR of 1.57 means that a DGF recipient has a hazard of dying that is 1.57 higher than non-DGF recipients, when considered among recipients who were alive and who did not experience graft failure at that time.

For the purposes of prognosis and medical decision-making, the primary interest is in the absolute risks of the event of interest; therefore, the subdistribution hazards model would be more relevant [46]. This competing risk analysis allows splitting the contribution of a covariate of each event type separately. For our example, the effect of DGF did not reach conventional significance. Furthermore, the estimated effect (sHR = 1.22, $P = 0.28$) was smaller than the corresponding DGF effect obtained by standard Cox analysis (csHR = 1.57). The major advantage of the competing risks approach is that the effects of each risk factor can be estimated and formally compared across different end points.

The conflicting findings of the impact of DGF on graft and patient survival results not only from the ambiguity in the definition of DGF but also from the statistical methodology used to study its effect. The impact of DGF on two types of graft loss was assessed in this study using specific methods designed for the competing risks analysis and was compared with the results of the standard survival analysis methods. Accounting for the Fine and Gray model, DGF was not significantly associated with patient death. However, it has a significant adverse effect on the hazard of graft failure, independent of AR. The results stress the importance of using appropriate statistical methods if competing risks are present.

This article also presents an overview of competing risks concepts in the context of KTx, including the bias in the standard Kaplan–Meier estimator. Competing risks are clearly important for medical research, and their negligence has important clinical implications. The *naive* interpretation of Kaplan–Meier estimates in the presence of competing risks as estimates of actual risks leads to potential overestimation of the actuals probabilities of graft failure and patient death and overestimation and inappropriate risk stratification in prognostic models. This is markedly important in a field such as kidney transplantation, where changes in survival-influencing factors, such as immunosuppression practices, organ allocation policies, or surgical techniques, may occur rapidly and where competing events are pervasive.

Authorship

IF, LT, JM, ACH and DM: participated in the design and coordination of the study and drafted the manuscript. IF, LT, JM and DM: conducted the biostatistical analyses. LSM, LD and ACH: were involved in the data collection and management of the study. Each author contributed important intellectual content during the drafting and revision of the manuscript and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved. All of the authors read, revised, and approved the final manuscript.

Funding

No funding.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Cox proportional hazard regression (cause-specific hazard model) for all possible events.

Table S2. Fine and Gray model (hazard of the subdistribution model) for all possible events.

References

1. Kyllonen LE, Salmela KT, Eklund BH, *et al.* Long-term results of 1047 cadaveric kidney transplantations with

special emphasis on initial graft function and rejection. *Transpl Int* 2000; **13**: 122.
2. Ojo AO, Hanson JA, Wolfe RA, Leichtman AB, Agodoa LY, Port FK. Long-term survival in renal transplant recipients with graft function. *Kidney Int* 2000; **57**: 307.
3. Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed graft function: risk factors and implications for renal allograft survival. *Transplantation* 1997; **63**: 968.
4. Ponticelli C. Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. *Nephrol Dial Transplant* 2014; **29**: 1134.
5. Gentil MA, Alcaide MP, Algarra GR, *et al.* Impact of delayed graft function on cadaveric kidney transplant outcome. *Transplant Proc* 2003; **35**: 689.
6. Marcen R, Orofino L, Pascual J, *et al.* Delayed graft function does not reduce the survival of renal transplant allografts. *Transplantation* 1998; **66**: 461.
7. Troppmann C, Gillingham KJ, Benedetti E, *et al.* Delayed graft function, acute rejection, and outcome after cadaver renal transplantation. The multivariate analysis. *Transplantation* 1995; **59**: 962.
8. Troppmann C, Gillingham KJ, Gruessner RW, *et al.* Delayed graft function in the absence of rejection has no long-term impact. A study of cadaver kidney recipients with good graft function at 1 year after transplantation. *Transplantation* 1996; **61**: 1331.
9. Butala NM, Reese PP, Doshi MD, Parikh CR. Is delayed graft function causally associated with long-term outcomes after kidney transplantation? Instrumental variable analysis. *Transplantation* 2013; **95**: 1008.
10. Ponticelli C, Villa M, Cesana B, Montagnino G, Tarantino A. Risk factors for late kidney allograft failure. *Kidney Int* 2002; **62**: 1848.
11. Feldman HI, Gayner R, Berlin JA, *et al.* Delayed function reduces renal allograft survival independent of acute rejection. *Nephrol Dial Transplant* 1996; **11**: 1306.
12. Giral-Classe M, Hourmant M, Cantarovich D, *et al.* Delayed graft function of more than six days strongly decreases long-term survival of transplanted kidneys. *Kidney Int* 1998; **54**: 972.
13. Boom H, Mallat MJ, de Fijter JW, Zwinderman AH, Paul LC. Delayed graft function influences renal function, but not survival. *Kidney Int* 2000; **58**: 859.
14. Tapiawala SN, Tinckam KJ, Cardella CJ, *et al.* Delayed graft function and the risk for death with a functioning graft. *J Am Soc Nephrol* 2010; **21**: 153.
15. Narayanan R, Cardella CJ, Cattran DC, *et al.* Delayed graft function and the risk of death with graft function in living donor kidney transplant recipients. *Am J Kidney Dis* 2010; **56**: 961.
16. Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. *Am J Transplant* 2011; **11**: 2279.
17. Perez Fontan M, Rodriquez-Carmona A, Bouza P, *et al.* Outcome of grafts with long-lasting delayed function after renal transplantation. *Transplantation*. 1996; **62**: 42.
18. Collett D. *Modelling Survival Data in Medical Research*. Chapman & Hall, London, 1994; XVII + 347.
19. Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med* 1999; **18**: 695.
20. Pintilie M. *Competing risks - A practical perspective*. Lda: John Wiley & Sons, 2006.
21. Southern DA, Faris PD, Brant R, *et al.* Kaplan-Meier methods yielded misleading results in competing risk scenarios. *J Clin Epidemiol* 2006; **59**: 1110.
22. Latouche A, Allignol A, Beyersmann J, Labopin M, Fine JP. A competing risks analysis should report results on all cause-specific hazards and cumulative incidence functions. *J Clin Epidemiol* 2013; **66**: 648.
23. Noordzij M, Leffondre K, van Stralen KJ, Zoccali C, Dekker FW, Jager KJ. When do we need competing risks methods for survival analysis in nephrology? *Nephrol Dial Transplant* 2013; **28**: 2670.
24. Pintilie M. Analysing and interpreting competing risk data. *Stat Med* 2007; **26**: 1360.
25. Moeschberger ML, Tordoff KP, Kochar N. A Review of Statistical Analyses for Competing Risks. In: Rao CR, Miller JP, Rao DC eds. *Handbook of Statistics: Epidemiology and Medical Statistics*. Amsterdam: Elsevier, 2008: 321–41.
26. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risks. *J Am Stat Assoc* 1999; **94**: 496.
27. Kim HT. Cumulative incidence in competing risks data and competing risks regression analysis. *Clin Cancer Res* 2007; **13**: 559.
28. Andersen PK, Geskus RB, de Witte T, Putter H. Competing risks in epidemiology: possibilities and pitfalls. *Int J Epidemiol* 2012; **41**: 861.
29. Ferrari P, Lim W, Dent H, McDonald SP. Effect of donor-recipient age difference on graft function and survival in live-donor kidney transplantation. *Nephrol Dial Transplant* 2011; **26**: 702.
30. Barbour SJ, Er L, Djurdjev O, Karim M, Levin A. Differences in progression of CKD and mortality amongst Caucasian, Oriental Asian and South Asian CKD patients. *Nephrol Dial Transplant* 2010; **25**: 3663.
31. Teixeira L, Rodrigues A, Carvalho MJ, Cabrita A, Mendonca D. Modelling competing risks in nephrology research: an example in peritoneal dialysis. *BMC Nephrol* 2013; **14**: 110.
32. Sapir-Pichhadze R, Young A. Joseph Kim S. Living donor age and kidney transplant outcomes: an assessment of risk across the age continuum. *Transpl Int* 2013; **26**: 493.
33. Verduijn M, Grootendorst DC, Dekker FW, Jager KJ, le Cessie S. The analysis of competing events like cause-specific mortality–beware of the Kaplan-Meier method. *Nephrol Dial Transplant* 2011; **26**: 56.

34. Gjertson DW, Dabrowska DM, Cui X, Cecka JM. Four causes of cadaveric kidney transplant failure: a competing risk analysis. *Am J Transplant* 2002; **2**: 84.
35. Holme I, Fellstrom BC, Jardine AG, Hartmann A, Holdaas H. Model comparisons of competing risk and recurrent events for graft failure in renal transplant recipients. *Clin J Am Soc Nephrol* 2013; **8**: 241.
36. Sud M, Tangri N, Levin A, Pintilie M, Levey AS, Naimark DM. CKD stage at nephrology referral and factors influencing the risks of ESRD and death. *Am J Kidney Dis* 2014; **63**: 928.
37. Haynes R, Staplin N, Emberson J, *et al.* Evaluating the Contribution of the Cause of Kidney Disease to Prognosis in CKD: results From the Study of Heart and Renal Protection (SHARP). *Am J Kidney Dis* 2014; **64**: 40.
38. Thompson CA, Zhang ZF, Arah OA. Competing risk bias to explain the inverse relationship between smoking and malignant melanoma. *Eur J Epidemiol* 2013; **28**: 557.
39. Lee YJ, Chang JH, Choi HN, *et al.* Donor-recipient age difference and graft survival in living donor kidney transplantation. *Transplant Proc.* 2012; **44**: 270.
40. Shin M, Park JB, Kwon CH, Joh JW, Lee SK, Kim SJ. Enhanced significance of donor-recipient age gradient as a prognostic factor of graft outcome in living donor kidney transplantation. *World J Surg* 2013; **37**: 1718.
41. Grunkemeier GL, Jin R, Eijkemans MJ, Takkenberg JJ. Actual and actuarial probabilities of competing risks: apples and lemons. *Ann Thorac Surg* 2007; **83**: 1586.
42. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. *Stat Med* 2007; **26**: 2389.
43. Lunn M, McNeil D. Applying Cox regression to competing risks. *Biometrics* 1995; **51**: 524.
44. Koller MT, Raatz H, Steyerberg EW, Wolbers M. Competing risks and the clinical community: irrelevance or ignorance? *Stat Med* 2012; **31**: 1089.
45. Lau B, Cole SR, Gange SJ. Competing risk regression models for epidemiologic data. *Am J Epidemiol* 2009; **170**: 244.
46. Wolbers M, Koller MT, Witteman JC, Steyerberg EW. Prognostic models with competing risks: methods and application to coronary risk prediction. *Epidemiology* 2009; **20**: 555.

Table S1. Cox proportional hazard regression (cause-specific hazard model) for all possible events

		Unadjusted models			Adjusted model (n=1127)		
		csHR	95% CI	p value	csHR	95% CI	p value
Graft Failure (censored for patient death)	**DGF-AR** (reference: non-DGF and non-AR)						
	Only DGF	1.79	1.28 - 2.48	0.001	1.50	1.06 - 2.13	0.023
	Only AR	2.99	2.17 - 4.10	<0.001	2.50	1.79 – 3.50	<0.001
	DGF + AR	3.37	2.35 - 4.84	<0.001	2.28	1.55 - 3.36	<0.001
	Transplant Era (reference: Era 4 > 2000)						
	Era 1 (< 1990)	2.58	1.65 - 4.03	<0.001	2.94	1.66 – 5.21	<0.001
	Era 2 (1990-1995)	2.03	0.34 - 3.09	0.001	2.73	1.60 – 4.67	<0.001
	Era 3 (1996-2000)	0.92	0.57 - 1.49	0.74	1.32	0.73 – 2.36	0.360
	ECD (yes vs. no)	2.41	1.73 - 3.34	<0.001	1.60	1.09 – 2.35	0.015
	Donor-recipient age difference (reference: 1st Q: < - 15 yr)						
	2nd Q (≥ -15 and ≤ -4 yr)	1.76	1.16 - 2.69	0.009	1.88	1.21 - 2.94	0.005
	3rd Q (> - 4 and ≤ +6 yr)	2.35	1.56 - 3.53	<0.001	2.09	1.36 - 3.22	0.001
	4th Q (> + 6 yr)	3.91	2.66 - 5.77	<0.001	3.18	2.06 - 4.92	<0.001
	Cause of ESRD (reference: others)						
	Diabetic nephropathy	1.71	1.01 - 2.89	0.046	2.55	1.44 - 4.52	0.001
	Glomerulonephritis	1.12	0.87 - 1.45	0.38	1.03	0.79 - 1.35	0.819
	Time on dialysis (>5 vs. <5 yr)	1.24	0.93 - 1.65	0.14	1.51	1.13 - 2.04	0.006
		Unadjusted models			Adjusted model (n=1182)		
		csHR	95% CI	p value	csHR	95% CI	p value
Patient Death (censored for graft failure)	**DGF-AR** (reference: non-DGF and non-AR)						
	Only DGF	1.95	1.41 - 2.68	<0.001	1.50	1.08 - 2.09	0.017
	Only AR	1.29	0.85 - 1.95	0.23	1.03	0.66 - 1.62	0.90
	DGF + AR	2.36	1.57 - 3.54	<0.001	1.49	0.94 - 2.37	0.094
	Transplant Era (reference: Era 4 > 2000)						
	Era 1 (< 1990)	2.63	1.69 - 4.08	<0.001	2.66	1.53 - 4.64	0.001
	Era 2 (1990-1995)	1.39	0.90 - 2.14	0.139	1.57	0.97 - 2.55	0.069
	Era 3 (1996-2000)	0.73	0.45 - 1.20	0.214	0.81	0.48 - 1.36	0.42
	Cause of ESRD (reference: others)						
	Diabetic nephropathy	2.56	1.60 - 4.11	<0.001	3.41	2.04 - 5.69	<0.001
	Glomerulonephritis	0.76	0.57 - 1.02	0.071	0.92	0.67 - 1.26	0.61
	HCV infection (positive vs. negative)	1.87	1.32 - 2.65	<0.001	1.56	1.08 - 2.25	0.017
	Recipient age (1 yr increase)	1.04	1.02 - 1.05	<0.001	1.06	1.04 - 1.07	<0.001

csHR=cause-specific hazard ratio; ERSD= end-stage renal disease; ECD= expanded-criteria donors; HCV=Hepatitis C virus; yr= year; Q= quartile. The bold printed covariables indicate statistical significance in the multivariable model.

Table S2. Fine and Gray model (hazard of the subdistribution model) for all possible events

		Unadjusted models			Adjusted model (n=1127)		
		sHR	95% CI	p value	sHR	95% CI	p value
Graft	**DGF-AR** (reference: non-DGF and non-AR)						
Failure	Only DGF	1.69	1.21 - 2.35	0.002	1.54	1.07 - 2.15	0.01
	Only AR	3.09	2.24 - 4.27	<0.001	2.53	1.84 - 3.48	<0.001
	DGF + AR	3.26	2.28 - 4.67	<0.001	2.30	1.57 - 3.38	<0.001
	Transplant Era (reference: Era 4 > 2000)						
	Era 1 (< 1990)	2.69	1.74 - 4.18	<0.001	2.10	1.32 - 3.35	0.002
	Era 2 (1990-1995)	2.23	1.49 - 3.33	0.001	2.08	1.38 - 3.14	<0.001
	Era 3 (1996-2000)	1.02	0.64 - 1.65	0.93	1.00	0.62 - 1.63	0.99
	ECD (yes vs. no)	2.10	1.51 - 2.92	<0.001	1.47	1.01 - 2.16	0.047
	Donor-recipient age difference (reference: 1st Q: < - 15 yr)						
	2nd Q (≥ -15 and ≤ -4 yr)	1.82	1.20 - 2.76	0.005	1.67	1.09 - 2.56	0.018
	3rd Q (> - 4 and ≤ +6 yr)	2.36	1.60 - 3.50	<0.001	1.92	1.28 - 2.87	0.001
	4th Q (> + 6 yr)	4.06	2.79 - 5.90	<0.001	2.97	1.98 - 4.45	<0.001
	Cause of ESRD (reference: others)						
	Diabetic nephropathy	1.71	1.01 - 2.89	0.046	2.11	1.22 - 3.65	0.008
	Glomerulonephritis	1.12	0.87 - 1.45	0.38	1.12	0.86 - 1.45	0.40
		Unadjusted models			Adjusted model (n=1182)		
		sHR	95% CI	p value	sHR	95% CI	p value
Patient	DGF-AR (reference: non-DGF and non-AR)						
Death	Only DGF	1.53	1.08 - 2.19	0.018	1.24	0.87 - 1.77	0.23
	Only AR	0.80	0.49 - 1.30	0.370	0.88	0.53 - 1.44	0.61
	DGF + AR	1.29	0.78 - 2.15	0.320	1.10	0.64 - 1.90	0.72
	Transplant Era (reference: Era 4 > 2000)						
	Era 1 (< 1990)	1.80	1.09 - 2.95	0.021	3.73	2.10 - 6.62	<0.001
	Era 2 (1990-1995)	1.29	0.81 - 2.04	0.280	2.01	1.23 - 3.30	0.006
	Era 3 (1996-2000)	0.75	0.44 - 1.27	0.280	0.98	0.57 - 1.67	0.93
	ECD (yes vs. no)	1.14	0.70 - 1.85	0.60			
	Donor-recipient age difference (reference: 4th Q: > +6 yr)						
	1st Q (< -15 yr)	3.07	1.90 - 4.95	<0.001	1.47		
	2rd Q (≥ -15 and ≤ -4 yr)	1.71	1.01 - 2.88	0.044			
	3rd Q (> - 4 and ≤ +6 yr)	1.82	1.07 - 3.10	0.027			
	Cause of ESRD (reference: others)						
	Diabetic nephropathy	1.41	0.75 - 2.64	0.28	1.76	0.92 - 3.36	0.088
	Glomerulonephritis	0.62	0.44 - 0.88	0.007	0.77	0.55 - 1.08	0.14

sHR=subdistribution hazard ratio; ERSD= end-stage renal disease; HCV=Hepatitis C virus; yr= year; Q=quartile. The bold printed covariables indicate statistical significance in the multivariable model.

.

4.7. REVIEW ARTICLE

BIOMARKERS IN KIDNEY TRANSPLANTATION: TRANSLATING TO CLINICAL PRACTICE

Fonseca. I. Biomarkers in kidney transplantation: Translating to clinical practice (Review Article). *Port J Nephrol Hypert* 2013; 27: 143-151

Improving long-term graft survival is a major challenge in kidney transplantation. Delayed graft function complicates the post-transplant management and has a negative impact on both the short and long-term outcomes. Numerous biomarkers in kidney transplantation have been evaluated in the past decade, but evidence to support their use in routine practice is currently limited.

This review was undertaken to discuss the current status of three biomarkers for the early diagnosis and prognosis of delayed graft function, namely uNGAL, oxidative stress and CysC and to reflect on our own interpretation and experience.

REVIEW ARTICLE

Port J Nephrol Hypert 2013; 27(3): 143-151
Advance Access publication 30 August 2013

Biomarkers in Kidney Transplantation: Translating to clinical practice

Biomarcadores em Transplantação Renal: do laboratório à prática clínica

Isabel Fonseca

Department of Nephrology and Kidney Transplantation
Centro Hospitalar do Porto, Hospital de Santo António, Porto, Portugal

Received for publication: 31/07/2013
Accepted: 05/08/2013

ABSTRACT

Improving long-term graft survival is a major challenge in kidney transplantation. Ischaemia-reperfusion injury is a critical early allograft insult that enhances the risk of delayed graft function, which is common in deceased-donor transplantation. Delayed graft function complicates the post-transplant management and has a negative impact on both short and long-term outcomes. The development of effective interventions to prevent and attenuate the injury caused by ischaemia-reperfusion is constricted by the limited ability of early detection of kidney damage. In recent years, clinical and translational research has focused on improvements in the diagnosis of acute kidney injury and provided prognostic information that is helpful in the post-transplant care. Numerous biomarkers in kidney transplantation have been evaluated in the past decade, but, so far, evidence to support their use in routine practice is limited. The purpose of this review is to examine the current status of three biomarkers for early diagnosis and prognosis of delayed graft function, namely urinary neutrophil gelatinase-associated lipocalin, oxidative stress and cystatin C. In addition, the concept of a biomarker is addressed, as well as the existing challenges and perspectives for developing a biomarker. This review discusses current literature and reflects the author's own interpretation and experience.

Keywords: Biomarker, Delayed graft function, Kidney transplantation

RESUMO

Melhorar a sobrevivência do transplante renal a longo prazo é um desafio. A lesão provocada pela isquemia e reperfusão constitui uma agressão precoce do enxerto renal e aumenta o risco de atraso de função do enxerto, que é comum no transplante de dador cadáver. A ocorrência de atraso de função do

Isabel Fonseca

enxerto condiciona a evolução do pós-transplante e tem um impacto negativo nos resultados imediatos e a longo prazo do transplante renal. O desenvolvimento de intervenções eficazes na prevenção e atenuação da lesão causada pelo processo de isquemia-reperfusão do órgão transplantado tem estado limitado pela ausência de marcadores precoces da lesão e disfunção renal.

Nos últimos anos, a investigação clínica e de translação tem conseguido melhorar a capacidade de diagnóstico da lesão aguda do enxerto renal e fornecido alguma informação de prognóstico, que pode ser útil no seguimento pós-transplante. Nas últimas décadas têm sido investigados inúmeros biomarcadores no transplante renal, mas a sua translação para a prática clínica não tem sido escassa. Esta revisão tem como objetivo a descrição do contexto atual de três biomarcadores para o diagnóstico precoce de atraso na função do enxerto: a lipocalina associada à gelatinase dos neutrófilos (NGAL), o stress oxidativo e a cistatina C. Adicionalmente serão abordados alguns conceitos básicos e as perpetivas de desenvolvimento de um biomarcador, com base numa revisão da literatura e na interpretação e experiencia pessoal de uma investigação em curso nessa área.

Palavras-chave: Atraso de função do enxerto, Biomarcadores, Transplante Renal

INTRODUCTION

Late failure of kidney transplants remains an important clinical problem and one of the leading causes of end-stage renal disease. Despite significant improvements in one-year kidney allograft survival, the rate of chronic graft loss after the first year remains significant and the actual kidney allograft half-life only showed a marginal improvement over the past decade[1]. The reasons for this slight improvement remain unclear. It is possible that some important determinants of long-term graft survival may not have changed sufficiently to improve the overall outcomes of kidney transplantation[2]. Patient death with a functioning allograft and chronic allograft failure are the two major causes of late transplant loss. The causes of chronic allograft failure are multifactorial and influenced by numerous immunological and non-immunological factors[1,2]. Generally, kidney transplants stabilize after recovering from the stress of implantation until declining of graft function due to specific diseases or conditions, such as recurrent renal disease, antibody-mediated rejection or a common process involving interstitial fibrosis and tubular atrophy the entity encompassed by the previous descriptive term "chronic allograft nephropathy", and more recently simply 'fibrosis/atrophy'[2,3].

Improving long-term graft survival is a major challenge in kidney transplantation. A patient submitted to a renal transplant would wonder if his or her transplanted kidney will work well and how long will it last. When will it be possible to identify valuable markers to distinguish patients at increased risk of graft dysfunction or of losing their transplant? Can biomarkers signal early transplant dysfunction, a process that is often undetectable? Can biomarkers help clinicians fine-tune their prognoses?

As in every other domain in medicine, in organ transplantation early diagnosis and timely intervention will improve outcomes. Clinicians need and continually look for tools to aid them on clinical assessment and to enhance their ability to identify the "vulnerable" patient at risk for graft dysfunction. Biomarkers are one such tool. They will allow to better identify high-risk individuals, to diagnose dysfunction promptly and accurately, and to effectively prognosticate outcomes and treat patients with a tailored and more individualized intervention.

WHAT IS A BIOMARKER?

Biomarker is a very broad term that can be used to describe any indicator of a biological state. The term biomarker, or biological marker, was introduced in 1989 as a Medical Subject Heading (MeSH) term and it was defined as a "*measurable and quantifiable*

biological parameters (eg, specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances), which serve as indices for health- and physiology-related assessments." More recently, in 2001, the definition was standardized by the Biomarker Definitions Working Group[4] as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention*". In practice, biomarkers can improve understanding about a disease and provide new knowledge of pathological mechanisms, making possible the earlier diagnosis and the delivery of more efficacious and safer therapies. Presently, it is not well established how biomarkers are categorized. Within the field of health care, biological markers are commonly classified based on the sequence of events from exposure to disease[5]: *biomarkers of exposure*, which are used in risk prediction, and *biomarkers of disease*, which are used in screening, diagnosis and prognosis.

To be clinically useful and prevent false-positive screening tests, a biological marker should be highly sensitive and specific in detecting disease or any other outcome. Regardless of the purpose for its use, it should be accurate, reproducible and standardized across different clinical units. Furthermore, it should be easily measured in a standard biological source (as blood or urine), obtained from a person (as blood pressure or electrocardiogram), or image-based (echocardiogram or computerized tomography scan), so that the information would be readily available and easy to interpret by clinicians[5,6]. In summary and according to the Food and Drug Administration, an ideal biomarker should be specific, sensitive, predictive, robust, simple, accurate, and inexpensive.

BIOMARKERS IN KIDNEY TRANSPLANTATION

In organ transplantation, initial graft dysfunction is one of the most important early post-operative problems, mainly due to the unavoidable ischemia-reperfusion injury that occurs in the transplanted organ. In kidney transplantation, ischemic injury of the renal allograft is a critical early insult that augments the risk of acute tubular necrosis and long-term graft loss[7,8]. The development of effective interventions is constricted by the limited ability of early detection of graft dysfunction. Current clinical indicators of kidney injury, like serum creatinine, are inadequate for timely diagnosis and prognosis. Thus, application of biomarkers in the field of kidney transplantation will allow to detect incipient graft dysfunction or rejection, will refine diagnoses and enable more effective post-transplant management, and thereby potentially improve short-term (e.g., delayed graft function, acute rejection) and long-term (e.g., allograft failure) outcomes.

Discovery of biomarkers is expanding at an unprecedented rate. Numerous biomarkers in kidney transplantation have been evaluated in the past decade, but, so far, evidence to support their use in routine practice is limited. In this article, we review the promising role of three biomarkers of delayed graft dysfunction, namely, neutrophil gelatinase-associated lipocalin, oxidative stress, and cystatin C.

NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN

Neutrophil gelatinase-associated lipocalin (NGAL) is one the most promising biomarkers of acute kidney injury in a variety of acute clinical settings[9-11]. Neutrophil gelatinase-associated lipocalin, also known as human neutrophil lipocalin or lipocalin-2, is a 25-kDa glycoprotein belonging to the lipocalin family and originally it was found covalently bounded to gelatinase in activated neutrophils. This lipocalin behaves as a bacteriostatic agent in acute infections and it is released to blood from activated neutrophils during inflammatory processes. It was demonstrated that NGAL exists in two separate body pools, the systemic and the renal one. In the systemic pool, NGAL is normally expressed at very low concentrations in several human tissues. After glomerular filtration, circulating NGAL is reabsorbed in the proximal tubules, catabolized and finally released with urine in small quantities. In the renal pool, NGAL is rapidly released from renal tubular cells in response to various insults to the kidney. Thus, in steady situations, NGAL is found in urine only in trace. In contrast, when massive NGAL quantities are expressed and excreted in urine this usually indicates injury and damage of proximal tubular cells, due to

Isabel Fonseca

ischaemia-reperfusion injury, hypoxia, nephrotoxins or chronic progressive changes[12,13].

Ischaemia-reperfusion injury is an inevitable consequence of kidney transplantation procedure and can be considered a form of post-transplantation acute kidney injury. For this reason, several studies investigated the utility of NGAL for the diagnostic and prognostic of acute graft dysfunction following kidney transplantation, with promising results[14-19]. Recently, the prognostic value of NGAL on graft function at one year post-transplantation was also examined[18,20].

The larger study on this subject is from Hollmen and colleagues[18]. These researchers demonstrated that urinary NGAL (uNGAL) is an early predictor of delayed graft function (DGF) following renal transplantation, in a prospective cohort study of 176 adult recipients transplanted with deceased-donor kidneys. Urine was collected before transplant, at then at days 1, 3, 7 and 14, and NGAL was measured at each time point. The uNGAL measured in the first morning following transplantation predicted DGF (defined as the need for dialysis during the first week after transplantation), particularly in cases where early graft function was expected on the basis of diuresis and decreasing plasma creatinine concentration. Patients who needed dialysis in the first post-transplant week had a slower decrease in uNGAL compared with recipients without DGF, and levels of uNGAL at day-1 predicted DGF (area under the curve (AUC) of 0.75). In 15 of 112 cases with urine output higher than 1L at day-1, uNGAL was a predictor of DGF, as well in 19 of 86 cases with a day-1 decrease in creatinine over than 1 mg/dl (AUC 0.74). Other authors showed also prominent results. Parikh *et al.* in a prospective study that included 53 consecutive patients undergoing living or deceased-donor transplantation (children and adults), measured NGAL in urine samples collected within the first 24 hours after transplantation and reported an AUC-ROC of 0.9 in predicting DGF[14]. Hall and coworkers evaluated uNGAL within 6h after transplantation in a 91-patient cohort of adults with a kidney graft from a deceased-donor, and predicted subsequent DGF with an AUC-ROC of 0.81[16].

Across a range of clinical studies, both urine and plasma NGAL has been shown to be a useful discriminatory marker of renal injury and an early predictor of DGF, with a performance greater than serum creatinine, the most commonly used surrogate measurement of glomerular filtration rate. In our experience, and similarly to other authors[14,18], we have chosen to measure NGAL in urine (uNGAL), instead of blood, since uNGAL represents tubule damage in the kidney rather than filtration from blood[13,21]. Although plasma NGAL is freely filtered by the glomerulus, it is largely reabsorbed in proximal tubules by efficient megalin-dependent endocytosis[11]. Thus, any urinary excretion of NGAL is likely only when there is concomitant proximal renal tubular injury that precludes NGAL reabsorption and/or increases *de novo* NGAL synthesis. Accordingly, an increased level of NGAL in urine usually indicates injury and damage of tubular cells and seems to be more specific compared to serum NGAL, which can be produced by other organs and released into the circulation following a transplant surgery[21]. The non-invasive nature of sample collection and the reduced number of interfering proteins were also other advantages taken in account when we choose to measure this biomarker in urine[10].

However, despite the undoubtedly value of urinary markers of kidney injury, their use in transplant recipients can be also a drawback because of possible transient graft anuria, which may preclude the availability of urine and consequently the lack of sample to measure NGAL. The persistent urine production by the native kidneys and the usual fluctuations of hydration status in these patients can also induce potential changes in urinary biomarker concentration, which can be another inconvenience to measure NGAL in urine. The genesis and sources of plasma and urinary NGAL require further clarification. However, despite the uncertainty of whether NGAL level performs better in urine or plasma/serum, both plasma/serum and urine NGAL levels appear to perform similarly well and provide a relevant advantage compared with serum creatinine, which is an insensible marker of kidney injury[9].

OXIDATIVE STRESS

Oxidative stress is one of the most important components of the ischaemia-reperfusion process[22-24]. It reflects an imbalance between reactive oxygen species (ROS) and cellular mechanisms for detoxifying

the reactive intermediates or for repairing the resulting damage. Disturbances in the normal state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell.

Oxygen free radicals or, more generally, ROS are products of normal cellular metabolism. It has been estimated that the average person has around 10,000–20,000 free radicals attacking each body cell every day. Free radicals are defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals, which gives a considerable reactivity to the free radical. The well-known radicals derived from oxygen, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) represent the most important class of radical species generated in living systems[25].

In physiological conditions, ROS are produced specifically to serve essential biological functions, as in defence against infections. In these conditions, the rates of free radical production and elimination are equal, leading to a steady state that is presumably tolerated by the cell. The antioxidant defence mechanisms can be divided into two major groups: endogenous, mainly enzymes, such as superoxide dismutases (SOD), catalases, glutathione reductases (GR) and peroxidases (GPx) and small molecules, mostly exogenous, acting as free radical scavengers (vitamins A, C, and E, carotenoids and polyphenol)[25]. In some pathological conditions, an imbalance between ROS generation and antioxidant capacity leads to enhanced ROS activity and oxidative stress. When these antioxidant mechanisms cannot counterbalance the amount of free radicals generated, cell damage and tissue injury can take place[26].

Reactive oxygen species may cause tissue injury via several mechanisms. As they are potent oxidizing and reducing agents, ROS directly damage cellular membranes and modify several biological molecules, such as lipids, proteins, and nucleic acids. The by-products of these reactions can serve as biomarkers of oxidative stress[22,23]. Of the many biological targets of oxidative stress, lipids are the most involved class of biomolecules. Lipid oxidation generates a huge variety of secondary products, including reactive carbonyl compounds, such as malondialdehyde (MDA). This aldehyde is the principal and most studied product of polyunsaturated fatty acid peroxidation, and for this reason MDA is a marker widely used to assess lipid peroxidation[25,26].

Markers of oxidative stress, including elevated levels of MDA and reduced antioxidant activity have been reported in renal patients[27,28]. There is huge amount of literature concerning oxidative stress and renal disease but data about kidney transplantation in the early stages are scarce. The restoration of kidney function after transplantation can lead to improvement of the oxidative stress[29], but some studies report increased systemic biomarkers of oxidative stress in kidney transplant recipients[30,31], specifically in the early phase of transplantation[32,33] and, thereafter, coexisting with chronic allograft tubular atrophy/interstitial fibrosis[30,34].

It has long been suspected that oxidative stress contributes to injury of ischaemic and reperfused tissues. In the setting of kidney transplantation, not only are there ischaemic and reperfusion periods obligated by the preservation and implantation procedures, but placement of the kidney into an immune milieu can also act as an adjuvant for oxidative damage. Reactive oxygen species are generated during both phases of ischaemia-reperfusion. As in other clinical conditions, if the scavenging capacity of kidney is beneath the excessive ROS generated, such oxidative imbalance may trigger a robust inflammatory response within the transplanted organ and lead to cellular destruction, tissue damage and graft dysfunction[33,35]. Thus, severe reperfusion injury is a risk factor for DGF and detection of ROS could be an early warning of graft injury. Waller and coworkers studied blood samples in porcine kidney allografts before and after reperfusion injury and demonstrated that both plasma carbonyl and 8-isoprostane (product of protein and lipid damage by free radicals respectively) could be reliable biomarkers to predict the reperfusion injury[36]. To the author's knowledge, no similar studies were done on this topic in human kidney transplantation.

A wide range of antioxidant enzymes may potentially exert a protective influence by limiting the production of ROS and the damage of oxidative stress following ischaemia-reperfusion injury of kidney graft. Conflicting results are reported in the literature on the activities of antioxidant enzymes in kidney transplant patients. Glutathione compounds and SOD have been reported to increase[37,38] decrease[31] or not

Isabel Fonseca

change[39] following renal transplantation. Whitin *et al.*[37] reported a rapid increase in plasma GPx activity after transplantation. The plasma GPx activity was two times higher 3 days after transplantation in adult patients who received a kidney transplant from a related donor; and rapidly increased over the first 2 weeks post-transplant in adult recipients from a deceased-donor and paediatric patients undergoing kidney transplantation from related donors. Zachara *et al.*[38] have shown that plasma GPx activity increased rapidly 3 days after renal transplantation, and doubled two weeks later. Both of these studies suggested that monitoring plasma GPx might be a useful marker for monitoring transplanted kidney function and a valuable tool for post-operative detection of DGF.

Not only in the early post-transplant period but also at longer-term, oxidants and antioxidants can be as biomarkers of graft dysfunction with diagnostic accuracy. Oxidative stress is believed to be a common pathway that leads to both immunological and non-immunological stress in the setting of kidney transplantation and to the development or progression of chronic allograft nephropathy. Increased plasma and intragraft levels of MDA and reduced antioxidant activity were found in kidney allografts with chronic tubular atrophy/interstitial fibrosis, which suggests the possibility of early detection, even when graft dysfunction is undetectable by serum creatinine[30,34,40].

The understanding about oxidative stress significantly advanced in the last decade, but these experimentally derived ideas have yet to be fully integrated into clinical practice. General evidence for involvement of ROS in hypoxia-reoxygenation injury includes detection of lipid peroxidation. Malondialdehyde is an end-product of lipid peroxidation and it is a frequently measured biomarker of oxidative stress. Studies on this topic are limited in kidney transplantation. Therefore, more research is needed to clearly define the role and clinical value of MDA and other oxidative stress markers in kidney transplantation.

CYSTATIN C

Cystatin C (CysC) is a low molecular mass protein that is produced at a constant rate by nearly all-human nucleated cells. This cystatin is freely filtered through the normal glomerular membrane, almost completely reabsorbed and degraded by proximal tubular cells, but it is not secreted by the tubules. Although its clearance cannot be measured because of this catabolism, its plasma or serum concentration has been shown to be independent of muscular mass, inflammatory diseases, sex, age or diet, and these properties make it a good measure of glomerular filtration rate (GFR) compared to the traditional measurement of creatinine[41,42]. As a result of this finding, several prediction equations have been derived from both paediatric and adult patients to estimate GFR from the serum CysC concentration[43,44]. Most of the studies that compared CysC levels or CysC derived equations with gold standard methods found CystC to be superior or, at least, equivalent to serum creatinine[42]. Some studies on selected patient groups, whose muscle mass is either reduced or undergoes rapid changes, also demonstrated CysC as a sensitive marker of GFR and independently of body composition[41].

Renal transplant recipients are a target group for whom precise determination of GFR is crucial. Allograft function following renal transplantation is commonly monitored using serum creatinine. However, plasma creatinine is far from being an ideal marker of GFR, despite its convenience and low cost. Since the first publication in 1998[45], quite a few original clinical papers have addressed the question of the use of CysC in kidney transplantation. A good number of studies identified serum CysC (or CysC-based equations) as a promising, easily measurable marker to estimate GFR with a higher diagnostic value than serum creatinine (or creatinine-based equations) and 24-hour creatinine clearance for evaluation of GFR in the follow-up of adult kidney transplant patients[46-48]. Very recently, Masson *et al.*[48] validated both of CysC-based CKD-EPI equations (2012) in 670 kidney transplant recipients and concluded that both performed better than the serum creatinine-based CKD-EPI equation (2009).

Glucocorticoid medication can be shortcoming in using serum CysC in this population and it is important to take into account when interpreting this serum marker. Glucocorticoid therapy is one of the few circumstances identified that have an impact on the production of CysC in a dose-dependent manner, leading to systematic underestimation of GFR[49]. Very

large doses of glucocorticoids have been described to increase the production of CysC[49], whereas low and medium doses of glucocorticoids do not seem to alter the production of CysC[50]. This, however, does not preclude the use of CysC in detecting impaired renal function in renal transplant patients on glucocorticoids, given that many studies have shown CysC to be still significantly more accurate in detecting impaired renal function in this patient group[49,50].

For kidney recipient follow-up, the ability to detect rapid changes in GFR is clinically more important than accuracy itself. For this reason, and due to the promising findings of CysC in kidney transplantation, the role of this marker in detecting post-transplant renal damage earlier than serum creatinine has been investigated[51-54]. Thervet *et al.*[51] in a prospective study of 30 renal transplant patients found that CysC allowed earlier diagnosis of renal function recovery than serum creatinine, particularly in patients with DGF. In a prospective study that included 30 consecutive patients with end-stage renal disease undergoing renal transplantation, Le Bricon *et al.* evaluated CysC as a marker of allograft function during the early post-operative transplantation period. Serum CysC was more sensitive than serum creatinine for detecting decreases in GFR and predicting DGF. Furthermore, a more prominent rise in plasma CycC values allowed a more rapid diagnosis of acute rejection or treatment nephrotoxicity, with the potential for more timely intervention[52]. Hall *et al.* also confirmed these findings in a cohort of 78 deceased-donor renal recipients and shown that CysC outperformed serum creatinine as a predictor of poor early graft function and the need for dialysis within the first week of kidney transplantation. The authors also proved that CysC was a good prognostic marker of graft function at 3 months[53]. In a recent article published in June 2012, Liu *et al.* evaluated the clinical value of CysC for the diagnosis of an acute rejection episode after renal transplantation in 76 recipients and concluded that CysC can predict acute rejection episode after renal transplantation[55]. Urine CysC was also studied recently by Hall *et al.*[54] in a prospective multicenter study that included 91 deceased-donor kidneys transplants. Serial urine samples were collected for 2 days following transplant and on the first post-operative day urine CysC was a predictor of DGF and of 3-month allograft function.

Cystatin C displays several good characteristics that make it a viable biomarker for early detection of DGF. Of the three markers addressed in this review, serum CysC is probably the biomarker most used and closest to the clinical validation in kidney transplantation.

THE AUTHOR'S EXPERIENCE

The Renal Transplant Unit at the Centro Hospitalar do Porto conducted a prospective, longitudinal study in 40 consecutive end-stage renal disease patients undergoing living or deceased-donor kidney transplantation, from December 2010 to May 2011. This study aimed to identify early markers of graft dysfunction in the peritransplant period and investigate their accuracy in predicting DGF (defined as dialysis requirement within the first week after transplantation). The receiver operating characteristic curve analysis is commonly used to evaluate biomarker utility in clinical diagnosis of disease, especially during biomarker development research. Using this statistical tool, the present study demonstrated uNGAL, MDA and CysC as good diagnostic markers on identifying patients with graft dysfunction in the early post-transplant period and who required dialysis in the first week (*articles submitted or in draft*). When analyzed separately, all three biomarkers predicted who would develop DGF with about the same degree of accuracy, and all of them with a diagnostic performance superior to serum creatinine.

Despite these encouraging results, this is not enough to certify any of these markers as diagnostic and prognostic biomarkers with wide clinical utility. Before each of these (or any other) biomarkers can be deployed in the clinic, they have to be repeatedly tested in hundreds of patients to assure that they serve as effective markers of acute graft dysfunction and prognostic indicators of dialysis-based DGF. It is a long and laborious pathway from identifying to validating a reliable biomarker.

Delayed graft function is a common complication affecting renal grafts immediately after transplantation. Since DGF has so many detrimental effects, accurate and early identification of features of DGF is remarkably important because it would allow more targeted and personalised treatment approaches.

Several studies were done in renal transplantation to identify biomarkers for the diagnosis of DGF. However, there is still no routine application of any of these markers in clinical transplantation. The first step was taken in the long march to translate a biomarker from the laboratory into the clinical practice. Generation of prospective data will now be necessary for validation and demonstration of the clinical utility of these markers in other centres and transplant recipients, across different practices and sets of variables. If validated, these biomarkers will be a major advantage for transplant recipients by allowing their care team to detect acute kidney injury before the risk of graft dysfunction becomes too high and the possibility of intervention less effective.

CONCLUSIONS

In renal transplant patients, early detection of impaired kidney function is critical so that efforts to prevent further deterioration of graft function or rejection can be instituted. Biomarker investigations are now an integral part of clinical research. The overall expectation of a biomarker is to enhance the ability to detect earlier an ongoing biological process and predict which patients will respond better to which interventions. To bring biomarkers to the clinic, it is mandatory to show a useful clinical application that is supported by the validation data. In the field of kidney transplantation, some biomarkers have successively gone the process of discovery and of validation, but fall short in their ability to contribute decisively to patient care. In a time of greater economic constraints and more personalized medicine, biomarkers are certain to have a presence in transplant care and coordinated and collaborative efforts should be made to implement novel biomarkers into the clinical practice.

Conflict of interest: None declared.

References

1. Meier-Kriesche HU, Schold JD, Kaplan B. Long-term renal allograft survival: Have we made significant progress or is it time to rethink our analytic and therapeutic strategies? Am J Transplant 2004;4(8):1289–1295
2. El Zoghby ZM, Stegall MD, Lager DJ, *et al.* Identifying specific causes of kidney allograft loss. Am J Transplant 2009;9(3):527–535
3. Solez K, Colvin RB, Racusen LC, *et al.* Banff '05 Meeting Report: Differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant 2007;7(3):518-526
4. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. Clin Pharmacol Ther 2001;69 (3):89–95
5. Perera FP, Weinstein IB. Molecular epidemiology: recent advances and future directions. Carcinogenesis 2000;21(3):517–524
6. Mayeux R. Biomarkers: Potential Uses and Limitations. NeuroRx 2004;1(2):182–188
7. Mas VR, Mueller TF, Archer KJ, Maluf DG. Identifying biomarkers as diagnostic tools in kidney transplantation. Expert Rev Mol Diagn 2011;11(2):183-196
8. Roedder S, Vitalone M, Kathri P, Sarwal MM. Biomarkers in solid organ transplantation: establishing personalized transplantation medicine. Genome Med 2011;3(6): 37
9. Haase M, Bellomo R, Devarajan P, Haase-Fielitz A; NGAL Meta-analysis Investigator Group. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. Am J Kidney Dis 54 2009;54(6):1012-1024
10. Devarajan P. Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomark Med 2010;4(2):265-280
11. Haase M, Devarajan P, Haase-Fielitz A, *et al.* The outcome of neutrophil gelatinase-associated lipocalin-positive subclinical acute kidney injury: a multicenter pooled analysis of prospective studies. J Am Coll Cardiol 2011;57(17):1752-1761
12. Schmidt-Ott KM, Mori K, Li JY, *et al.* Dual action of neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol 2007;18(2):407-413
13. Mori K, Lee HT, Rapoport D, *et al.* Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest 2005; 115(3):610-621
14. Parikh CR, Jani A, Mishra J, *et al.* Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant 2006;6(7):1639-1645
15. Kusaka M, Kuroyanagi Y, Mori T, *et al.* Serum neutrophil gelatinase-associated lipocalin as a predictor of organ recovery from delayed graft function after kidney transplantation from donors after cardiac death. Cell Transplant 2008;17(1-2):129-134
16. Hall IE, Yarlagadda SG, Coca SG, *et al.* IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation. J Am Soc Nephrol 2010;21(1):189-197
17. Bataille A, Abbas S, Semoun O, *et al.* Plasma neutrophil gelatinase-associated lipocalin in kidney transplantation and early renal function prediction. Transplantation 2011;92(9):1024-1030
18. Hollmen ME, Kyllonen LE, Inkinen KA, Lalla ML, Salmela KT. Urine neutrophil gelatinase-associated lipocalin is a marker of graft recovery after kidney transplantation. Kidney Int 2011;79(1):89-98
19. Magnusson NE, Hornum M, Jorgensen KA, *et al.* Plasma neutrophil gelatinase associated lipocalin (NGAL) is associated with kidney function in uraemic patients before and after kidney transplantation. BMC Nephrol 2012;13:8
20. Hall IE, Doshi MD, Reese PP, Marcus RJ, Thiessen-Philbrook H, Parikh CR. Association between peritransplant kidney injury biomarkers and 1-year allograft outcomes. Clin J Am Soc Nephrol 2012;7(8):1224-1233
21. Kuwabara T, Mori K, Mukoyama M, *et al.* Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. Kidney Int 2009;75(3):285-294
22. Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am J Physiol Cell Physiol 2002;282(2):C227-241
23. Eltzschig HK, Collard CD. Vascular ischaemia and reperfusion injury. Br Med Bull 2004;70:71-86
24. Dolegowska B, Blogowski W, Domański L. Association between the perioperative antioxidative ability of platelets and early post-transplant function of kidney allografts: a pilot study. PLoS One 2012;7(1):e29779

25. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 2004;266(1-2):37-56

26. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006;160(1):1-40

27. Oberg BP, McMenamin E, Lucas FL, *et al.* Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. Kidney Int 2004;65(3):1009-1016

28. Mimic-Oka J, Simic T, Djukanovic L, Reljic Z, Davicevic Z. Alteration in plasma antioxidant capacity in various degrees of chronic renal failure. Clin Nephrol 1999;51(4):233-241

29. Simmons EM, Langone A, Sezer MT, *et al.* Effect of renal transplantation on biomarkers of inflammation and oxidative stress in end-stage renal disease patients. Transplantation 2005;79(8):914-919

30. Simic-Ogrizovic S, Simic T, Reljic Z, *et al.* Markers of oxidative stress after renal transplantation. Transpl Int 1998;11 (Suppl 1):S125-129

31. Campise M, Bamonti F, Novembrino C, *et al.* Oxidative stress in kidney transplant patients. Transplantation 2003;76(10):1474-1478

32. Zahmatkesh M, Kadkhodaee M, Mahdavi-Mazdeh M, *et al.* Oxidative stress status in renal transplant recipients. Exp Clin Transplant 2010;8(1):38-44

33. Ardalan MR, Estakhri R, Hajipour B, *et al.* Erythropoietin ameliorates oxidative stress and tissue injury following renal ischaemia/reperfusion in rat kidney and lung. Med Princ Pract 2013;22(1):70-74

34. Djamali A. Oxidative stress as a common pathway to chronic tubulointerstitial injury in kidney allografts. Am J Physiol Renal Physiol 2007;293(2):F445-455

35. Hariharan N, Zhai P, Sadoshima J.Oxidative stress stimulates autophagic flux during ischemia/reperfusion. Antioxid Redox Signal 2011;14(11):2179-2190

36. Waller HL, Harper SJ, Hosgood SA, *et al.* Biomarkers of oxidative damage to predict ischaemia-reperfusion injury in an isolated organ perfusion model of the transplanted kidney. Free Radic Res 2006;40:1218-1225

37. Whitin JC, Tham DM, Bhamre S, *et al.* Plasma glutathione peroxidase and its relationship to renal proximal tubule function. Mol Genet Metab 1998;65(3):238-245

38. Zachara BA, Wlodarczyk Z, Masztalerz M, Adamowicz A, Gromadzinska J, Wasowicz W. Selenium concentrations and glutathione peroxidase activities in blood of patients before and after allogenic kidney transplantation. Biol Trace Elem Res 2004;97(1):1-13

39. Vostálová J, Galandáková A, Svobodová AR, *et al.* Time-course evaluation of oxidative stress-related biomarkers after renal transplantation. Ren Fail 2012;34(4):413-419

40. Djamali A, Reese S, Yracheta J, Oberley T, Hullett D, Becker B. Epithelial-to-mesenchymal transition and oxidative stress in chronic allograft nephropathy. Am J Transplant 2005;5(3):500-509

41. Filler G, Bökenkamp A, Hofmann W, Le Bricon T, Martínez-Brú C, Grubb A. Cystatin C as a marker of GFR-history, indications, and future research. Clin Biochem 2005;38(1):1-8

42. Chew JS, Saleem M, Florkowski CM, George PM. Cystatin C – A paradigm of evidence based laboratory medicine. Clin Biochem Rev 2008;29(2):47-61

43. Le Bricon T, Thervet E, Froissart M, *et al.* Plasma cystatin C is superior to 24-h creatinine clearance and plasma creatinine for estimation of glomerular filtration rate 3 months after kidney transplantation. Clin Chem 2000;46(8):1206–1207

44. Hoek FJ, Kemperman FA, Krediet RT. A comparison between cystatin C, plasma creatinine and the Cockcroft and Gault formula for the estimation of glomerular filtration rate. Nephrol Dial Transplant 2003;18(10):2024–2031

45. Plebani M, Dall'Amico R, Mussap M, *et al.* Is serum cystatin C a sensitive marker of glomerular filtration rate (GFR)? A preliminary study on renal transplant patients. Ren Fail 1998;20(2):303–309

46. Herget-Rosenthal S, Trabold S, Huesing J, Heemann U, Philipp T, Kribben A. Cystatin C – An accurate marker of glomerular filtration rate after renal transplantation? Transpl Int 2000;13(4):285-289.

47. Malheiro J, Fonseca I, Martins LS, *et al.* A comparison between serum creatinine and cystatin C-based equations for estimation of graft function. Transplant Proc 2012;44(8):2352-2356

48. Masson I, Maillard N, Tack I, *et al.* GFR estimation using standardized cystatin C in kidney transplant recipients. Am J Kidney Dis 2013;61(2):279-284

49. Risch L, Herklotz R, Blumberg A, Huber AR. Effects of glucocorticoid immunosuppression on serum cystatin C concentrations in renal transplant patients. Clin Chem 2001;47(11):2055–2059

50. Bökenkamp A, van Wijk JA, Lentze MJ, Stoffel-Wagner B. Effect of corticosteroid therapy on serum cystatin C and beta2-microglobulin concentrations. Clin Chem 2002;48(7):1123-1126

51. Thervet E, Le Bricon T, Hugot V, *et al.* Early diagnosis of renal function recovery by cystatin C in renal allograft recipients. Transplant Proc 2000;32(8):2779

52. Le Bricon T, Thervet E, Benlakehal M, Bousquet B, Legendre C, Erlich D. Changes in plasma cystatin C after renal transplantation and acute rejection in adults. Clin Chem 1999;45(12):2243–2249

53. Hall IE, Doshi MD, Poggio ED, Parikh CR. A comparison of alternative serum biomarkers with creatinine for predicting allograft function after kidney transplantation. Transplantation 2011;91(1): 48-56

54. Hall IE, Koyner JL, Doshi MD, Marcus RJ, Parikh CR. Urine cystatin C as a biomarker of proximal tubular function immediately after kidney transplantation. Am J Nephrol 2011;33(5):407-413

55. Liu J. Evaluation of serum cystatin C for diagnosis of acute rejection after renal transplantation. Transplant Proc. 2012;44(5):1250-1253

Correspondence to:
Drª Isabel Fonseca
Department of Nephrology and Kidney Transplantation
Centro Hospitalar do Porto, Hospital de Santo António
Largo Prof. Abel Salazar
4099-001 Porto, Portugal
E-mail: isabelf27@gmail.com

Chapter 5

Discussion

Content

DISCUSSION

Transplantation is a revolutionary opportunity for end-stage renal disease patients to regain their normal daily life. During its history the surgical technique of transplantation became technically straightforward.[276] In the current era, modern anesthesia and fine surgical materials support the surgical procedure, and the advances in postoperative immunosuppression in the past 20 years have attenuated the early-risk period and had a positive impact on short and long-term survival. Despite these progresses, a gradual but inevitable decline in allograft function at two or more years after transplantation is observed in approximately half of the patients who receive a kidney allograft transplant and these patients will lose their renal allografts within 10 to 12 years after transplantation because of the loss of functioning.[2, 277-279]

We reviewed data from our kidney transplant experience over the past three decades and regarding the primary adult recipients transplanted from a deceased-donor with at least one-year of functioning; the main cause of late transplant loss was chronic allograft failure in approximately 61% of all of the graft losses. In 2013, 450 kidney transplantations were performed in Portugal. Currently, more than 1900 patients are waiting for an organ transplant, and each month approximately 30 patients are added to the waiting list. A significant number of patients on the waiting list are added as a result of the functional failure of a first transplant, which reflects our current inability to ensure long-term allograft function and survival and represents a major problem in transplantation medicine.

The time to failure of a renal allograft is greatly determined by the initial function achieved after transplantation, the number and severity of insults to the graft, and a number of tissue characteristics. The inevitable ischemia-reperfusion injury, DGF occurrence, acute rejection episodes, drug-related nephrotoxicity, and de novo allosensitization to the donor are some of the insults that can occur after kidney transplantation. The individual susceptibility to injury and the ability of the tissue to repair the damages are other important characteristics that also affect graft survival.

Delayed graft function is a clinical diagnosis that describes the acute transplant kidney dysfunction that occurs in the immediate postoperative phase after the transplantation procedure, which adversely impacts both short- and long-term renal allograft function.[26, 30] Several studies have demonstrated that DGF of the kidney results in an increased length of hospital stay and costs, an increased incidence of acute rejection and an inferior graft survival following kidney transplantation.[24, 30, 32, 40, 280-283] The role of DGF in long-term graft and patient survival has not reached a consensus. Some single-center studies have reported that DGF without rejection may have no impact on long-term graft survival;[273-275]

however, other investigators have demonstrated that DGF is associated with a poor graft outcome independent of rejection.[11, 32, 38] We evaluated the impact of DGF over the previous three decades of experience of our kidney transplant center using a competing events approach. We confirmed that DGF, independent of acute rejection, has a detrimental effect on long-term graft survival. After adjusting for most factors traditionally associated with graft failure, early kidney dysfunction has a clear adverse effect on long-term graft survival, meaning that the presence or absence of DGF will provide an indication of the life-expectancy of the kidney graft.

Some controversies also exist regarding the impact of DGF on patient survival, with several studies that support the adverse impact of DGF[26, 36-38] and other studies that refute this effect.[274, 284] Neither of these studies accounted for competing risks. In our study, using a cause-specific Cox proportional hazards regression model we confirmed the negative effect of DGF on patient outcome, but not when the analysis was based on the cumulative incidence of the failure types (Fine and Gray models). Both approaches are valid and the choice of the appropriate approach depends on the aim of the study (etiology vs. prediction).[266, 270, 272] If the primary interest is focused on the etiological question of how the covariates affect the event of interest, the cause-specific hazard models would be most appropriate because they directly model the covariate effect on event rates in subjects at risk.[285] Using this approach, our study showed that DGF significantly increased the risk of mortality (csHR=1.57, *P*=0.029). This hazard can be interpreted among the recipients who did not experience the event of interest (patient death), i. e., the recipients that were censored because they are alive or were previously transferred for dialysis because of graft failure (competing event). Considering our study, the csHR of 1.57 indicates that a DGF recipient has a hazard of dying 1.57 higher than non-DGF recipients, among recipients who were alive and that did not experience graft failure at the time. For the purposes of prognosis and medical decision-making, the primary interest is focused on the absolute risks of the event of interest; thus, the subdistribution hazards model (Fine and Gray model) would be more relevant.[286, 287] This competing risk analysis allows splitting the contribution of a covariate on each event type separately. For our study, the effect of DGF did not reach conventional significance and the estimated effect (sHR=1.22, P=0.28) was smaller than the corresponding DGF effect obtained by a cause-specific hazards model (csHR=1.57). The major advantage of the competing risks approach is that the effects of each risk factor can be estimated and formally compared across different endpoints. Graft failure and patient death are competing endpoints that are mutually exclusive. Therefore, because DGF is significantly associated with a higher probability of graft failure (the competing event is more frequent), the probability of experiencing an alternative competing risk (patient death) is lower and

the effect of DGF on patient survival is non-significant.
It appears that the conflicting findings of the impact of DGF on graft and patient survival result not only from the ambiguity in the definition of DGF but also from the statistical methodology used to study its effects. Considering the subdistribution hazard models, DGF was not significantly associated with patient death; however, it had a significant adverse effect on the hazard of graft failure independent of acute rejection.

We used a dialysis-based definition for DGF. There is no uniformly accepted definition for DGF; however, the dialysis requirement within the first postoperative week is likely the most common definition used.[288, 289] It is true that dialysis requirements may not reflect allograft dysfunction. Patients may need dialysis despite good graft function because of hyperkalemia or fluid overload. Thus, a new graft in these patients can be inaccurately classified as DGF. Similarly, recipients with significant residual renal function prior to transplantation can be misclassified as not having DGF despite a very low GFR in the allograft. In these patients, it is not possible to differentiate the urine output from the native kidney and the transplanted kidney, which hides the presence of DGF. Therefore, the need for renal replacement therapy can be subjective and represents a clinical-dependent decision, which is a clear limitation of this definition.
The other option is to use a creatinine-based definition for DGF. However, a post-transplant interpretation of allograft function based on serum creatinine can also be difficult. Patients are not in a steady state, and a substantial amount of renal mass can be dysfunctional without appreciable changes in the SCr, which misclassifies patients as not having DGF. Moreover, the frequently performed hemodialysis session immediately prior to surgery to normalize the potassium levels of the patient may falsely lower the post-transplant creatinine and hide a graft dysfunction, which also results in the misclassification of patients as not having DGF. Thus, a creatinine-based definition for DGF appears to be inadequate. Because the main cause of DGF is ischemia and reperfusion injury, which excludes other causes for DGF, such as acute rejection, calcineurin inhibitor toxicity and surgical complications we preferred to use a functional definition of DGF. Similar to other studies[66, 67, 69, 71, 72, 77, 290] we used a “harder” outcome to define DGF: the need for dialysis in the first week after kidney transplantation, which has recently been reinforced to be universally adopted as the clinical definition of DGF and as a study endpoint.[289]

The specific understanding of the importance of DGF in renal transplantation has long been hampered by the lack of specific and sensitive markers for this condition and the absence of studies with long-term follow-up. Long-term follow-up studies are difficult to achieve. Traditional study endpoints in kidney transplantation have focused on the first

year after transplantation. These endpoints are easier and more rapid to evaluate than the gold standard endpoints for long-term survival, which continues to be graft failure and patient death. Surrogate markers or endpoints can be used in place of conventional endpoints and represent a significant contribution to the early diagnosis, longitudinal prognoses, and outcome prediction. These surrogate markers or endpoints are used in *lieu* of the true endpoint to evaluate the outcome more rapidly, less expensively and/or less invasively.

In the transplant community, there has been a research focus on the identification of a biomarker that may serve as a surrogate for late graft loss.[291-293] A natural candidate for this surrogate endpoint is renal function. The creatinine levels and changes in the SCr levels within the first year post-transplantation are important parameters that influence and predict long-term graft survival.[292, 294-299] However, not all studies support the validity of SCr as a predictor of long-term graft loss.[45, 300, 301] As a result of this divergence and to proceed with the prospective study as planned, we examined the role of SCr in long-term graft survival at our center. Our results also highlighted that SCr at 1, 6, and 12 months as well as the changes between these months, had a significant relationship with the graft survival rate, which suggests that renal function within the first year after transplantation is an important parameter that influences long-term graft survival and is a reliable surrogate marker of late transplant outcome at our center.

One of the concerns in current transplant research is to obtain insight into the factors that are associated with long-term graft loss and to identify early markers of allograft dysfunction, as well as potential interventional pathways. Thus, special attention in this dissertation was focused on early biomarkers of DGF with the aim to improve the diagnosis and prediction of the long-term outcome not only for renal grafts but also for patients. The prospects of a patient who returns to dialysis after kidney transplantation are poor not only with respect to their quality of life but also for their survival.[302] Thus, nine candidate biomarkers were studied, including one biomarker in urine and eight biomarkers in blood. Some of these biomarkers were first examined by our group as early potential markers of kidney graft dysfunction.

Neutrophil gelatinase-associated lipocalin was the only biomarker that we measured in urine. As previously stated, when we designed this study to explore the usefulness of NGAL as a marker of graft functional recovery in the field of kidney transplantation, few studies on this topic were published in kidney transplantation settings at the time. Since then, more than twenty studies have been published, and most of them, similar to our study, have shown NGAL's significant predictivity for DGF.[69, 71, 72, 164, 166, 169-171] Across a range of clinical studies, both urine and serum NGAL have been shown to be useful

discriminatory markers of renal injury and early predictors of DGF with better performance than SCr, which is the most commonly used surrogate measurement of the GFR rate. An additional advantage is that uNGAL was independently and significantly associated with one-year graft function as evaluated by SCr, which we have confirmed to be a surrogate marker of long-term survival in our center. In addition to the research by our group, two other studies confirmed this association.[171, 172]

In our experience and similar to other authors,[71, 72, 164, 171] we have chosen to measure NGAL in urine instead of blood because uNGAL represents tubule damage in the kidney rather than filtration from the blood.[150, 303] Although plasma NGAL is freely filtered by the glomerulus, it is largely reabsorbed in the proximal tubules by efficient megalin-dependent endocytosis.[119] Thus, urinary excretion of NGAL likely occurs only when there is concomitant proximal renal tubular injury that precludes NGAL reabsorption and/or increases *de novo* NGAL synthesis. Accordingly, an increased level of NGAL in the urine typically indicates injury and damage of the tubular cells and appears to be more specific compared with serum NGAL, which can be produced by other organs and released into the circulation following a transplant surgery.[303] The non-invasive nature of sample collection and the reduced number of interfering proteins were other advantages considered when we chose to measure this biomarker in urine.[123]

However, despite the undoubted value of urinary markers of kidney injury, their use in transplant recipients can also be a drawback because of potential transient graft anuria, which may preclude the availability of urine and, consequently, result in the lack of sample material to measure NGAL. The persistent urine production by the native kidneys and the typical fluctuations in the hydration status in these patients can also induce potential changes in the urinary biomarker concentration, which can be another inconvenience in the measurement of NGAL in urine. The genesis and sources of plasma and urinary NGAL require further clarification. However, despite the uncertainty of whether the NGAL level performs better in the urine or plasma/serum, both plasma/serum and urine NGAL levels appear to perform similarly well and provide a relevant advantage compared with SCr, which is an insensitive marker of kidney injury.[61, 63, 304]

We also examined two adipokines as biomarkers of graft dysfunction. In chronic kidney disease, the clinical significance and prognostic implications of leptin and adiponectin are not well understood. To date, most of the research regarding leptin and adiponectin has focused on their associations with metabolic and cardiovascular health. To date and to the best of our knowledge, no reported study has examined the clinical utility of these two adipokines in the diagnosis of graft dysfunction after kidney transplantation. Thus, this study was designed based on the assumption that impaired clearance of leptin (and

adiponectin) could signal graft impairment earlier than SCr. Of the two adipokines measured, leptin most closely agreed with our initial hypothesis. The leptin levels shortly after transplantation and during the first week were anticipated to be significantly higher in patients who require dialysis, which suggests that higher leptinemia after kidney transplantation can reflect graft dysfunction. At day-1, leptinemia provided some albeit small, yet incremental prognostic information regarding the established marker. We expected that serum leptin would be rapidly cleared from circulation after kidney transplantation, faster than SCr, and anticipated a reduction of SCr in response to prompt graft function. It is possible that other factors that coexist in the immediate post-transplant period might stimulate leptin synthesis and delay its elimination.

Inflammation [305] has been implicated in augmentation of leptin secretion. Surgical stress is also associated with an increase in serum leptin concentration [306, 307]. Both of these conditions concur in kidney transplantation. Organ transplantation is a surgical procedure that involves an inevitable ischemia-reperfusion injury with a consequently deleterious activation of cellular oxidases that cause oxidative damage, tissue injury and inflammation.[308] Leptin is an acute phase reactant that is involved in the cytokine network of acute inflammation and the stress response.[306] It is possible that inflammatory cytokines that result from the organ transplant process can stimulate leptin synthesis and attenuate its clearance from circulation, primarily in recipients with graft dysfunction. These effects would also explain why the decrement of plasma leptin concentration in the immediate post-transplant period did not reach normal levels in most patients, even in the patients with prompt graft function. At day-7, 64% of our recipients still had levels that exceeded the upper limit of the leptin reference range; this percentage was 40% when only the recipients with prompt graft function were considered.

It is noteworthy that the separate analysis of male and female patients indicated that in the latter group, the performance of leptin was considerably better than when all subjects were combined. Unfortunately, the small number of female patients precluded a sensitive analysis in this gender. This finding also strengthens the importance of gender as a determinant variable for an individual's leptin status and the need for a stratified analysis of this variable. There is no uniformly accepted definition for "normal" leptin (or ADPN), and the classification of an individual's "leptin status" is hampered by lack of published reference ranges.[309] The current study reinforces that gender and body mass index are major determinants of circulating leptin levels and any attempt to provide ranges of expected leptin levels should account for at least these two variables.

Adiponectinemia was not significantly higher in the recipients with graft dysfunction and was not a predictor of DGF. At least during the first week, graft dysfunction did not reflect an impaired clearance of adiponectin, which suggests that factors other than renal

function may be involved. A recent study from Song and co-workers[310] demonstrated that a decline in circulating adiponectin levels during the initial 72 h after a subtotal nephrectomy in mice with renal failure was associated with the down regulation of adiponectin. According to this reasoning, we can also speculate that the decrease in the circulating adiponectin levels observed within the first week after kidney transplantation could be because of two different mechanisms according to graft function: an enhanced filtration of circulating adiponectin and urinary excretion in prompt graft patients and a decline in the local expression of adiponectin in the glomerular endothelium as a result of the amplified ischemia-reperfusion injury that typically characterizes DGF. Future studies on this topic are needed. Many factors remain unknown regarding adipokines in the field of kidney transplantation.

We reported the independent association of high levels of plasma MDA with DGF and with poorer one-year allograft function. To the best of our knowledge, this study is the first to demonstrate this association in kidney transplantation recipients. Malondialdehyde is a naturally occurring end product of lipid peroxidation and the most studied product of polyunsaturated fatty acid peroxidation; therefore, it has been frequently used as a biomarker of oxidative stress.[311-316] Oxidative stress is one of the most important components of the ischemia-reperfusion process,[176, 196, 317] which is an inevitable phenomenon in kidney transplantation. During ischemia-reperfusion, high levels of free radicals are formed and increased levels of lipid peroxidation (and MDA) are detected. Accordingly, we showed that elevated MDA levels reflected kidney dysfunction and predicted the need for renal replacement therapy within the first week following kidney transplantation better than SCr.[318]

A wide range of protective substances, such as antioxidant enzymes, may potentially exert a protective influence by limiting the production of ROS and the damage of oxidative stress following an ischemia-reperfusion injury of the kidney graft. Conflicting results have been reported regarding the activities of antioxidant enzymes in kidney transplantation patients. The levels of antioxidant enzymes have been reported to increase,[205, 206] decrease[183, 319] or not change[207, 320] following kidney transplantation. Compared with healthy controls, our patients had significantly higher levels of SOD and GR prior to kidney transplantation, which were most likely in response to the intensified oxidative stress of end-stage renal disease patients. However, no significant changes were observed following kidney transplantation even when stratified by graft function. Delayed graft function after kidney transplantation is a manifestation of acute kidney injury; thus, for an existing injury or one in progress, it is natural that higher quantities of ROS are released. Under normal conditions, endogenous antioxidant enzymes neutralize these radicals and

the enzyme activity subsequently decreases. Another possibility is that the capacity of the antioxidant defense system increases as a response to a higher production of oxidant radicals. Because neither of these two situations occurred, we hypothesized that in the first week after kidney transplantation, the antioxidant defense system does not effectively respond to the higher levels of oxidative stress detected in DGF. It is possible that similar to MnSOD, the major enzymes of the antioxidant system become inactivated during the early phase of ATP depletion that typically occurs in ischemia.[202]

Regarding CysC, which is the most widely used "novel" marker and a potential replacement for SCr, we can highlight the very good performance of this biomarker in the diagnosis of graft dysfunction and prediction of dialysis need within the first week after transplantation. It is well known that moderate and high dose glucocorticoids can limit the usefulness of CysC soon after kidney transplantation.[105] Nevertheless, we confirmed that CysC can signal graft dysfunction significantly better than SCr and we corroborated its performance not only as a single marker but also in combination with other markers (study V). This study summarizes the current usage of biomarker combinations for clinical purposes. The kidney transplant recipients and the graft injury process are complex and could not be deduced from the measurement of a single biomarker. Some biomarkers display low sensitivities or specificities, which prevent their translation to clinical practice. Therefore, combining biomarkers into a multimarker panel can improve the classification of patients and their clinical usefulness.

Thus, based on the biomarkers studied, we explored the optimal marker combination for DGF diagnosis and identified a simple, accurate and clinically applicable tool with CysC, MDA and SCr (AUC=0.96, sensitivity=100%; specificity=86%). We assumed that even if a highly sensitive marker was discovered, SCr would continue to be used because it is the well-established marker. Thus, new biomarkers were complemented with the current established marker to increment the diagnostic and prognostic value. The best performance was achieved with this triple-biomarker approach.

Although uNGAL has shown promising results as a predictor of DGF, the performance of the combinations that comprise uNGAL was lower than the triple-marker approach with SCr, MDA, and CysC. One potential explanation for the inferior performance of uNGAL might have been the lack of samples in some recipients with transient anuria. In our study, 5 recipients were anuric the morning after graft reperfusion, which resulted in 12.5% of our patients without urine samples for measurement of uNGAL at this particular time point. These patients could not be included in any of the approaches that included uNGAL, which reduced the sample size and the performance of the uNGAL combinations.

In kidney transplantation, numerous diagnostic biomarkers have been evaluated in the

previous decade; however, evidence to support their use in routine practice is currently limited. The discovery of novel biomarkers can be complex and costly. In this study, we demonstrated that a novel marker (MDA) predicted who would develop DGF with approximately the same degree of accuracy as serum CysC and that both had a diagnostic performance that was superior to SCr. Undoubtedly, CysC displays several good characteristics that make it a viable biomarker for the early detection of DGF. Nonetheless and particularly during the first week when high-doses of corticosteroids are used, glucocorticoid medication can compromise the use of serum CysC in kidney transplantation, and it is important to considerer this effect when interpreting this serum marker. Thus, a combination of biomarkers may be more valuable for the diagnosis of DGF and the prognosis of graft function. Because DGF is a critical early insult to the renal allograft, which augments the risk of long-term graft loss, and it is a complex process with multiple underlying pathogenic mechanisms and confounding risk factors, it would be prudent to predict DGF with more than a single biomarker, at least in some situations. Malondialdehyde represents a valuable marker as an alternative or as a complement in the risk prediction not only in relation to the serum CysC and any other serum/plasma markers but also for urine biomarkers such as NGAL, which cannot be measured if a urine sample cannot be obtained, particularly during the transient anuria that commonly occurs after kidney transplantation.

Chapter 6

Conclusions and Future Directions

Content

Concluding remarks
Future directions

CONCLUDING REMARKS

The work presented in this dissertation describes the investigation and examination of nine biomarkers for the prediction of DGF. The studies have shown promising results that indicate that some of the candidate biomarkers have potential for the early detection of DGF and one-year graft function and are feasible for use in the clinical setting.

As in every other field of medicine, early diagnosis and timely intervention will improve the outcomes of organ transplantation. Clinicians need and continually look for tools to aid them in clinical assessment and to enhance their ability to identify “vulnerable” patients who are at risk for graft dysfunction. Biomarkers are one such tool. They will enable us to better identify high-risk individuals, promptly diagnose dysfunction, accurately and effectively prognosticate outcomes, and treat patients with a tailored and more individualized intervention.

It would be ideal to identify a single marker with a very high discriminatory accuracy, defined in terms of the test sensitivity and specificity. However, for most diseases, and particularly for kidney transplantation, single markers might not reflect all of the facets of initial graft dysfunction, and they do not have adequate sensitivity or specificity for practical purposes. One approach to increase the clinical value of biomarkers and improve their screening sensitivity is to identify additional biomarkers and to use a combination of them because a multimarker strategy might better characterize the complexity of DGF.

During the research of this project several statistical tools were used and discussed, namely linear mixed models and survival analysis that accounted for competing risks. The reasons and advantages of using these methodologies were previously addressed; however, we should emphasize the importance of appropriate longitudinal and survival approaches in a field such as transplantation, where changes in survival-influencing factors, such as immunosuppression practices, organ allocation policies, or surgical techniques, may occur rapidly and where competing events are pervasive. Most studies in kidney transplantation use standard survival analysis methods, which treat patient death with a functioning graft as a censored event that is similar to patients lost to follow-up. However, this violates a principle of survival analysis, noninformative censoring, or the requirement that prognosis does not influence censorship. The predictors of graft failure are also shared with risk factors for death; thus, when patients are censored for death in traditional survival analyses, this relationship is neglected and it introduces bias into the overall analysis. Hence, a standard survival analysis is flawed and often overestimates the absolute risk in the presence of an informative competing event. Therefore, methods for competing risks should be used in transplant settings, with the choice of method guided

by the scientific question.

The key findings that resulted from this thesis research can be summarized as follows:

First-year renal function as a surrogate of long-term graft survival (Study I)

- Of the large number of variables that were examined, the SCr levels at 1, 6 and 12 months following kidney transplantation as well as the changes between 1 and 6 months and between 6 and 12 months were independently associated with late graft failure.
- These findings were important to establish SCr levels as a surrogate endpoint that will reflect long-term renal transplant outcomes and would be a reliable substitute for the subsequent prospective studies designed to identify earlier and more sensitive markers of graft dysfunction.

NGAL (Study II)

- Urinary NGAL appears to play a promising role as an early marker of graft functional recover and a predictor of DGF and one-year graft function.
- The kinetics of uNGAL levels following kidney transplantation change according to graft function: recipients with graft damage and who went on to develop DGF had initial higher levels of uNGAL that rose further on the subsequent post-transplant days, which differs from patients with prompt graft function.
- At each time-point measurement, 4 to 5 recipients were anuric, which resulted in approximately 12% of patients without a urine sample to determine uNGAL. Despite the clear value of uNGAL as a promising biomarker for allograft dysfunction, the use of a urinary marker in the perioperative period of kidney transplantation can also be a drawback because of the potential for transient graft anuria, which may preclude the availability of urine and consequently the lack of sample to measure NGAL.

Oxidative stress markers (Study III)

- Recipients who developed DGF presented increased MDA levels during the first week after kidney transplantation, which appear to reflect the postischemic tissue damage of DGF kidneys. Compared with pre-transplant, these patients presented higher MDA levels at 8-to-12 h following kidney transplantation, in contrast to recipients with prompt graft function whose MDA levels continuously decreased throughout the week.
- Malondialdehyde is an early marker of DGF; however, it also demonstrated its predictive value as early as 8-to-12 h following kidney transplantation in terms of the

evolution of graft function and the need for dialysis during the first week. In regards to clinical application, a new biomarker should be more accurate in the prediction of DGF than the current SCr.

- Levels of MDA at day-7 were one of the best predictors of one-year SCr: higher MDA levels on day-7 were associated with worse one-year graft function, which suggests that oxidative damage will reflect long-term injury.

Adipokines (Study IV)

- Circulating levels of leptin and adiponectin decrease after successful transplantation, although the values remained higher than the upper limits of the leptin and adiponectin reference ranges.
- During the first post-transplant week, the recipients with graft impairment had higher leptin levels; the decline of leptin levels, but not adiponectin, depends on the function of the kidney allograft.
- The leptin levels at day-1, but not adiponectin, slightly outperformed the traditional biomarker SCr in the assessment of DGF prognosis.

Biomarker combination (Study V)

- A combination of biomarkers outperformed SCr in the early diagnosis of DGF, and the best performance was achieved by a triple-marker approach (AUC = 0.96, sensitivity = 100%; specificity = 86%) that used SCr, MDA, and CysC, which can be simply measured in routine blood samples to result in easier clinical decision-making.

Competing risks (Study VI)

- In kidney transplantation, chronic graft failure and patient death with graft function are competing endpoints that are mutually exclusive.
- In the presence of competing risk events the complement of the Kaplan-Meier estimate (1-KM) wrongly overestimates the probability of occurrence of the event of interest.
- The application of a regression model for subdistribution hazard showed that DGF, alone and independent of acute rejection, has a significant detrimental effect on long-term graft survival but not on patient survival.
- The impact of DGF on long-term outcome is controversial. The different criteria used to define DGF, as well as the use of inappropriate statistical methods may be related to the divergence of opinions.

FUTURE DIRECTIONS

The results presented in this dissertation open a number of possibilities for future research. Although the results from this thesis have contributed to and improved some aspects of our understanding regarding several potential biomarkers of early graft dysfunction, there is still significant work to be completed prior to their application in routine clinical practice.

The literature is replete with proposed candidate biomarkers assessed in single cohorts; however, few biomarkers have been independently validated. This clearly highlights that biomarker development is a difficult venture and requires an iterative approach. Validation in an independent sample set and side-by-side evaluation of comparable biomarkers are essential to confirm or discard conclusions drawn from the discovery phase.

Malondialdehyde was the most promising novelty that arose from this work. The next step is obviously to validate this marker, alone or in combination with a triple-marker approach with SCr and CysC, in an independent cohort with a substantially larger data set, and, if possible across multiple institutions. There is still a long way to go from the identification of a new biomarker to the implementation in a routine clinical assay.

Prediction modeling takes multiple clinical and laboratory variables and converts them into a probability of disease. Nomograms and prognostic scores are increasingly utilized and practical tools that allows for the prediction of risk in clinical practice. These tools provide accurate individualized estimates of outcomes and summarize the amount of risk associated with each covariate of interest. Some prognostic scores have been previously published to predict DGF using several demographical and clinical variables. The addition of other prognostic markers, such as MDA and CysC, to the currently available nomograms might enhance predictive accuracy and refine management of recipients. Therefore, an important objective for future research is to construct a DGF nomogram including MDA and CysC and validate in an independent cohort as well as prospective analysis of patients undergoing kidney transplantation.

Interest regarding MDA as a biomarker in kidney transplantation was triggered by our findings and led to a recent invitation to write a chapter of a book on the subject titled “Malondialdehyde as a biomarker in kidney transplantation” *in* “Biomarkers in Diseases of Kidney: Methods, Discoveries and Applications”. It is known that oxygen free radicals are one of the most likely agents responsible for initiating the damage associated with reperfusion injury in renal transplantation and more research is needed to answer the questions that arise from this study. We showed that MDA was an early marker of graft

damage and a predictor of DGF. It seems that the elevation of MDA levels results from graft damage. However, are the higher levels of MDA pathophysiology involved in the development of graft loss in renal transplant recipient?

General evidence for the involvement of reactive oxygen species in hypoxia-reoxygenation injury includes the detection of lipid peroxidation. Studies on this topic are limited regarding renal recipients. Malondialdehyde is an end product of lipid peroxidation and a biomarker of oxidative stress and early graft dysfunction. The prognostic value of MDA levels at the seventh day for one-year graft function was independent of the other significant predictors of one-year graft function. However, the implications of the increased production of MDA in the progression of allograft dysfunction are unknown. Since our group of 40 kidney recipients is an ongoing cohort and the MDA results were the first to be published from this cohort, the prospective clinical follow-up of these 40 recipients will allow us to evaluate MDA levels after the first year of graft functioning and thereby investigate whether these elevated levels persist and are associated with chronic kidney dysfunction and graft failure.

In relation to the other biomarkers studied, the leptin results were the most intriguing. We focused our research in leptin as an early marker of graft dysfunction and we expected that serum leptin would be rapidly cleared from circulation after kidney transplantation, faster than SCr, and anticipate the reduction of SCr in response to prompt graft function. However, leptin levels only slightly outperformed SCr in identifying graft dysfunction. These results warrant further studies. Leptin is an acute phase reactant and other factors coexist in the immediate post-transplant period that can delay its elimination. Moreover, recent studies highlight an important and novel function for leptin: up-regulation of inflammatory immune responses. It will be interesting to investigate the possible impact of leptin in transplant rejection and its effect on boosting allo-immunity and not only as marker of kidney function.

Therefore, more research is needed. Further integration of the multiple levels of biological information will be needed to achieve this goal. We look forward to a time when transplant recipients, who have already suffered from end-stage renal failure, can enjoy a new lifetime with their allograft.

“Knowing is not enough; we must apply.

Willing is not enough, we must do”

Goethe

References

REFERENCES

1. Wolfe RA, Ashby VB, Milford EL*, et al.* (**1999**). Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. N Engl J Med 341**:** 1725-30.
2. Meier-Kriesche HU, Schold JD, Kaplan B. (**2004**). Long-term renal allograft survival: have we made significant progress or is it time to rethink our analytic and therapeutic strategies? Am J Transplant 4**:** 1289-95.
3. Pascual M, Theruvath T, Kawai T*, et al.* (**2002**). Strategies to improve long-term outcomes after renal transplantation. N Engl J Med 346**:** 580-90.
4. Marcen R, Fernandez-Rodriguez A, Rodriguez-Mendiola N*, et al.* (**2009**). Evolution of rejection rates and kidney graft survival: a historical analysis. Transplant Proc 41**:** 2357-9.
5. Chang SH, Russ GR, Chadban SJ*, et al.* (**2007**). Trends in kidney transplantation in Australia and New Zealand, 1993-2004. Transplantation 84**:** 611-8.
6. Lamb KE, Lodhi S, Meier-Kriesche HU. (**2011**). Long-term renal allograft survival in the United States: a critical reappraisal. Am J Transplant 11**:** 450-62.
7. Sellares J, de Freitas DG, Mengel M*, et al.* (**2012**). Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. Am J Transplant 12**:** 388-99.
8. Hariharan S, Johnson CP, Bresnahan BA*, et al.* (**2000**). Improved graft survival after renal transplantation in the United States, 1988 to 1996. N Engl J Med 342**:** 605-12.
9. El-Zoghby ZM, Stegall MD, Lager DJ*, et al.* (**2009**). Identifying specific causes of kidney allograft loss. Am J Transplant 9**:** 527-35.
10. Collins AJ, Foley RN, Herzog C*, et al.* (**2010**). Excerpts from the US Renal Data System 2009 Annual Data Report. Am J Kidney Dis 55**:** S1–S420.
11. Ponticelli C, Villa M, Cesana B*, et al.* (**2002**). Risk factors for late kidney allograft failure. Kidney Int 62**:** 1848-54.
12. Marcen R, Morales JM, Fernandez-Rodriguez A*, et al.* (**2010**). Long-term graft function changes in kidney transplant recipients. NDT Plus 3**:** ii2-ii8.
13. Solez K, Colvin RB, Racusen LC*, et al.* (**2007**). Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant 7**:** 518-26.
14. Einecke G, Sis B, Reeve J*, et al.* (**2009**). Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. Am J Transplant 9**:** 2520-31.
15. Briganti EM, Russ GR, McNeil JJ*, et al.* (**2002**). Risk of renal allograft loss from recurrent glomerulonephritis. N Engl J Med 347**:** 103-9.
16. de Vries AP, Bakker SJ, van Son WJ*, et al.* (**2004**). Metabolic syndrome is associated with impaired long-term renal allograft function; not all component criteria contribute equally. Am J Transplant 4**:** 1675-83.

17. Kreis HA, Ponticelli C. (**2001**). Causes of late renal allograft loss: chronic allograft dysfunction, death, and other factors. Transplantation 71**:** SS5-9.
18. Barba J, Zudaire JJ, Robles JE*, et al.* (**2013**). Complications of kidney transplantation with grafts from expanded criteria donors. World J Urol 31**:** 893-900.
19. Pascual J, Zamora J, Pirsch JD. (**2008**). A systematic review of kidney transplantation from expanded criteria donors. Am J Kidney Dis 52**:** 553-86.
20. Meier-Kriesche HU, Kaplan B. (**2002**). Waiting time on dialysis as the strongest modifiable risk factor for renal transplant outcomes: a paired donor kidney analysis. Transplantation 74**:** 1377-81.
21. Shoskes DA, Halloran PF. (**1996**). Delayed graft function in renal transplantation: etiology, management and long-term significance. J Urol 155**:** 1831-40.
22. Gill JS, Pereira BJ. (**2003**). Chronic kidney disease and the transplant recipient. Blood Purif 21**:** 137-42.
23. Perico N, Cattaneo D, Sayegh MH*, et al.* (**2004**). Delayed graft function in kidney transplantation. Lancet 364**:** 1814-27.
24. Yarlagadda SG, Klein CL, Jani A. (**2008**). Long-term renal outcomes after delayed graft function. Adv Chronic Kidney Dis 15**:** 248-56.
25. Schroppel B, Legendre C. (**2014**). Delayed kidney graft function: from mechanism to translation. Kidney Int 86**:** 251-8.
26. Siedlecki A, Irish W, Brennan DC. (**2011**). Delayed graft function in the kidney transplant. Am J Transplant 11**:** 2279-96.
27. Lehtonen SR, Isoniemi HM, Salmela KT*, et al.* (**1997**). Long-term graft outcome is not necessarily affected by delayed onset of graft function and early acute rejection. Transplantation 64**:** 103-7.
28. Troppmann C, Gillingham KJ, Benedetti E*, et al.* (**1995**). Delayed graft function, acute rejection, and outcome after cadaver renal transplantation. The multivariate analysis. Transplantation 59**:** 962-8.
29. Khalkhali HR, Ghafari A, Hajizadeh E*, et al.* (**2010**). Risk factors of long-term graft loss in renal transplant recipients with chronic allograft dysfunction. Exp Clin Transplant 8**:** 277-82.
30. Yarlagadda SG, Coca SG, Formica RN, Jr.*, et al.* (**2009**). Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. Nephrol Dial Transplant 24**:** 1039-47.
31. Nicholson ML, Wheatley TJ, Horsburgh T*, et al.* (**1996**). The relative influence of delayed graft function and acute rejection on renal transplant survival. Transpl Int 9**:** 415-9.
32. Ojo AO, Wolfe RA, Held PJ*, et al.* (**1997**). Delayed graft function: risk factors and implications for renal allograft survival. Transplantation 63**:** 968-74.
33. Pfaff WW, Howard RJ, Patton PR*, et al.* (**1998**). Delayed graft function after renal transplantation. Transplantation 65**:** 219-23.
34. Pirsch JD, Ploeg RJ, Gange S*, et al.* (**1996**). Determinants of graft survival after renal transplantation. Transplantation 61**:** 1581-6.

35. Johnston O, O'Kelly P, Spencer S*, et al.* (**2006**). Reduced graft function (with or without dialysis) vs immediate graft function--a comparison of long-term renal allograft survival. Nephrol Dial Transplant 21**:** 2270-4.
36. Tapiawala SN, Tinckam KJ, Cardella CJ*, et al.* (**2010**). Delayed graft function and the risk for death with a functioning graft. J Am Soc Nephrol 21**:** 153-61.
37. Narayanan R, Cardella CJ, Cattran DC*, et al.* (**2010**). Delayed graft function and the risk of death with graft function in living donor kidney transplant recipients. Am J Kidney Dis 56**:** 961-70.
38. Butala NM, Reese PP, Doshi MD*, et al.* (**2013**). Is delayed graft function causally associated with long-term outcomes after kidney transplantation? Instrumental variable analysis. Transplantation 95**:** 1008-14.
39. Sharif A, Borrows R. (**2013**). Delayed graft function after kidney transplantation: the clinical perspective. Am J Kidney Dis 62**:** 150-8.
40. Ponticelli C. (**2014**). Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. Nephrol Dial Transplant 29**:** 1134-40.
41. Seron D, Arns W, Chapman JR. (**2008**). Chronic allograft nephropathy--clinical guidance for early detection and early intervention strategies. Nephrol Dial Transplant 23**:** 2467-73.
42. Nankivell BJ, Chapman JR. (**2006**). Chronic allograft nephropathy: current concepts and future directions. Transplantation 81**:** 643-54.
43. Herrero-Fresneda I, Torras J, Cruzado JM*, et al.* (**2003**). Do alloreactivity and prolonged cold ischemia cause different elementary lesions in chronic allograft nephropathy? Am J Pathol 162**:** 127-37.
44. Fletcher JT, Nankivell BJ, Alexander SI. (**2009**). Chronic allograft nephropathy. Pediatr Nephrol 24**:** 1465-71.
45. Chapman JR, O'Connell PJ, Nankivell BJ. (**2005**). Chronic renal allograft dysfunction. J Am Soc Nephrol 16**:** 3015-26.
46. Campistol JM, Boletis IN, Dantal J*, et al.* (**2009**). Chronic allograft nephropathy--a clinical syndrome: early detection and the potential role of proliferation signal inhibitors. Clin Transplant 23**:** 769-77.
47. Group. BDW: Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. In *Clin Pharmacol Ther* (vol 69), 2001/03/10 ed, 2001, pp 89-95
48. De Gruttola VG, Clax P, DeMets DL*, et al.* (**2001**). Considerations in the evaluation of surrogate endpoints in clinical trials. summary of a National Institutes of Health workshop. Control Clin Trials 22**:** 485-502.
49. Vasan RS. (**2006**). Biomarkers of cardiovascular disease: molecular basis and practical considerations. Circulation 113**:** 2335-62.
50. Dickerson BC. (**2010**). Advances in quantitative magnetic resonance imaging-based biomarkers for Alzheimer disease. Alzheimers Res Ther 2**:** 21.
51. Mayeux R. (**2004**). Biomarkers: potential uses and limitations. NeuroRx 1**:** 182-8.

52. Flamenbaum M. (**2002**). Business strategy for implementation of biomarkers in drug development. Drug Discovery World Spring**:** 9-14.
53. Bodovitz S, Patterson SD. (**2003**). Protein biomarkers strategies. Drug Discovery World Fall**:** 67-78.
54. Lachenbruch PA, Rosenberg AS, Bonvini E*, et al.* (**2004**). Biomarkers and surrogate endpoints in renal transplantation: present status and considerations for clinical trial design. Am J Transplant 4**:** 451-7.
55. Frank R, Hargreaves R. (**2003**). Clinical biomarkers in drug discovery and development. Nat Rev Drug Discov 2**:** 566-80.
56. Brotman B, Prince AM. (**1988**). Gamma-glutamyltransferase as a potential surrogate marker for detection of the non-A, non-B carrier state. Vox Sang 54**:** 144-7.
57. Paone JF, Waalkes TP, Baker RR*, et al.* (**1980**). Serum UDP-galactosyl transferase as a potential biomarker for breast carcinoma. J Surg Oncol 15**:** 59-66.
58. Boone CW, Kelloff GJ. (**1993**). Intraepithelial neoplasia, surrogate endpoint biomarkers, and cancer chemoprevention. J Cell Biochem Suppl 17F**:** 37-48.
59. Prentice RL. (**1989**). Surrogate endpoints in clinical trials: definition and operational criteria. Stat Med 8**:** 431-40.
60. Lemley KV. (**2007**). An introduction to biomarkers: applications to chronic kidney disease. Pediatr Nephrol 22**:** 1849-59.
61. Slocum JL, Heung M, Pennathur S. (**2012**). Marking renal injury: can we move beyond serum creatinine? Transl Res 159**:** 277-89.
62. Mehta RL, Chertow GM. (**2003**). Acute renal failure definitions and classification: time for change? J Am Soc Nephrol 14**:** 2178-87.
63. Waikar SS, Betensky RA, Bonventre JV. (**2009**). Creatinine as the gold standard for kidney injury biomarker studies? Nephrol Dial Transplant 24**:** 3263-5.
64. Mas VR, Mueller TF, Archer KJ*, et al.* (**2011**). Identifying biomarkers as diagnostic tools in kidney transplantation. Expert Rev Mol Diagn 11**:** 183-96.
65. Roedder S, Vitalone M, Khatri P*, et al.* (**2011**). Biomarkers in solid organ transplantation: establishing personalized transplantation medicine. Genome Med 3**:** 37.
66. Pianta TJ, Peake PW, Pickering JW*, et al.* (**2014**). Clusterin in Kidney Transplantation: Novel Biomarkers Versus Serum Creatinine for Early Prediction of Delayed Graft Function. Transplantation.
67. Welberry Smith MP, Zougman A, Cairns DA*, et al.* (**2013**). Serum aminoacylase-1 is a novel biomarker with potential prognostic utility for long-term outcome in patients with delayed graft function following renal transplantation. Kidney Int 84**:** 1214-25.
68. Zaza G, Rascio F, Pontrelli P*, et al.* (**2014**). Karyopherins: potential biological elements involved in the delayed graft function in renal transplant recipients. BMC Med Genomics 7**:** 14.
69. Bataille A, Abbas S, Semoun O*, et al.* (**2011**). Plasma neutrophil gelatinase-associated lipocalin in kidney transplantation and early renal function prediction. Transplantation 92**:** 1024-30.

70. Fonseca I, Oliveira JC, Almeida M*, et al.* (**2013**). Neutrophil gelatinase-associated lipocalin in kidney transplantation is an early marker of graft dysfunction and is associated with one-year renal function. J Transplant 2013**:** 650123.
71. Hall IE, Yarlagadda SG, Coca SG*, et al.* (**2010**). IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation. J Am Soc Nephrol 21**:** 189-97.
72. Hollmen ME, Kyllonen LE, Inkinen KA*, et al.* (**2011**). Urine neutrophil gelatinase-associated lipocalin is a marker of graft recovery after kidney transplantation. Kidney Int 79**:** 89-98.
73. Hall IE, Bhangoo RS, Reese PP*, et al.* (**2014**). Glutathione S-transferase iso-enzymes in perfusate from pumped kidneys are associated with delayed graft function. Am J Transplant 14**:** 886-96.
74. Field M, Lowe D, Cobbold M*, et al.* (**2014**). The use of NGAL and IP-10 in the prediction of early acute rejection in highly sensitized patients following HLA-incompatible renal transplantation. Transpl Int 27**:** 362-70.
75. Yang J, Choi HM, Seo MY*, et al.* (**2014**). Urine liver-type fatty acid-binding protein predicts graft outcome up to 2 years after kidney transplantation. Transplant Proc 46**:** 376-80.
76. Stubendorff B, Finke S, Walter M*, et al.* (**2014**). Urine protein profiling identified alpha-1-microglobulin and haptoglobin as biomarkers for early diagnosis of acute allograft rejection following kidney transplantation. World J Urol.
77. Steubl D, Hettwer S, Vrijbloed W*, et al.* (**2013**). C-terminal agrin fragment--a new fast biomarker for kidney function in renal transplant recipients. Am J Nephrol 38**:** 501-8.
78. Abrahamson M, Barrett AJ, Salvesen G*, et al.* (**1986**). Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids. J Biol Chem 261**:** 11282-9.
79. Abrahamson M, Olafsson I, Palsdottir A*, et al.* (**1990**). Structure and expression of the human cystatin C gene. Biochem J 268**:** 287-94.
80. Chapman HA, Riese RJ, Shi GP. (**1997**). Emerging roles for cysteine proteases in human biology. Annu Rev Physiol 59**:** 63-88.
81. Abrahamson M, Grubb A, Olafsson I*, et al.* (**1987**). Molecular cloning and sequence analysis of cDNA coding for the precursor of the human cysteine proteinase inhibitor cystatin C. FEBS Lett 216**:** 229-33.
82. Shi GP, Sukhova GK, Grubb A*, et al.* (**1999**). Cystatin C deficiency in human atherosclerosis and aortic aneurysms. J Clin Invest 104**:** 1191-7.
83. Pierre P, Mellman I. (**1998**). Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. Cell 93**:** 1135-45.
84. Bjorck L, Grubb A, Kjellen L. (**1990**). Cystatin C, a human proteinase inhibitor, blocks replication of herpes simplex virus. J Virol 64**:** 941-3.
85. Taupin P, Ray J, Fischer WH*, et al.* (**2000**). FGF-2-responsive neural stem cell proliferation requires CCg, a novel autocrine/paracrine cofactor. Neuron 28**:** 385-97.
86. Tenstad O, Roald AB, Grubb A*, et al.* (**1996**). Renal handling of radiolabelled human cystatin C in the rat. Scand J Clin Lab Invest 56**:** 409-14.

87. Simonsen O, Grubb A, Thysell H. (**1985**). The blood serum concentration of cystatin C (gamma-trace) as a measure of the glomerular filtration rate. Scand J Clin Lab Invest 45**:** 97-101.
88. Dharnidharka VR, Kwon C, Stevens G. (**2002**). Serum cystatin C is superior to serum creatinine as a marker of kidney function: a meta-analysis. Am J Kidney Dis 40**:** 221-6.
89. Roos JF, Doust J, Tett SE*, et al.* (**2007**). Diagnostic accuracy of cystatin C compared to serum creatinine for the estimation of renal dysfunction in adults and children--a meta-analysis. Clin Biochem 40**:** 383-91.
90. Jacobsson B, Lignelid H, Bergerheim US. (**1995**). Transthyretin and cystatin C are catabolized in proximal tubular epithelial cells and the proteins are not useful as markers for renal cell carcinomas. Histopathology 26**:** 559-64.
91. Grubb A, Simonsen O, Sturfelt G*, et al.* (**1985**). Serum concentration of cystatin C, factor D and beta 2-microglobulin as a measure of glomerular filtration rate. Acta Med Scand 218**:** 499-503.
92. Filler G, Bokenkamp A, Hofmann W*, et al.* (**2005**). Cystatin C as a marker of GFR--history, indications, and future research. Clin Biochem 38**:** 1-8.
93. Chew JS, Saleem M, Florkowski CM*, et al.* (**2008**). Cystatin C--a paradigm of evidence based laboratory medicine. Clin Biochem Rev 29**:** 47-62.
94. Le Bricon T, Thervet E, Froissart M*, et al.* (**2000**). Plasma cystatin C is superior to 24-h creatinine clearance and plasma creatinine for estimation of glomerular filtration rate 3 months after kidney transplantation. Clin Chem 46**:** 1206-7.
95. Hoek FJ, Kemperman FA, Krediet RT. (**2003**). A comparison between cystatin C, plasma creatinine and the Cockcroft and Gault formula for the estimation of glomerular filtration rate. Nephrol Dial Transplant 18**:** 2024-31.
96. Plebani M, Dall'Amico R, Mussap M*, et al.* (**1998**). Is serum cystatin C a sensitive marker of glomerular filtration rate (GFR)? A preliminary study on renal transplant patients. Ren Fail 20**:** 303-9.
97. Herget-Rosenthal S, Trabold S, Huesing J*, et al.* (**2000**). Cystatin C--an accurate marker of glomerular filtration rate after renal transplantation? Transpl Int 13**:** 285-9.
98. Malheiro J, Fonseca I, Martins LS*, et al.* (**2012**). A comparison between serum creatinine and cystatin C-based equations for estimation of graft function. Transplant Proc 44**:** 2352-6.
99. Masson I, Maillard N, Tack I*, et al.* (**2013**). GFR estimation using standardized cystatin C in kidney transplant recipients. Am J Kidney Dis 61**:** 279-84.
100. Risch L, Herklotz R, Blumberg A*, et al.* (**2001**). Effects of glucocorticoid immunosuppression on serum cystatin C concentrations in renal transplant patients. Clin Chem 47**:** 2055-9.
101. Bjarnadottir M, Grubb A, Olafsson I. (**1995**). Promoter-mediated, dexamethasone-induced increase in cystatin C production by HeLa cells. Scand J Clin Lab Invest 55**:** 617-23.
102. Bokenkamp A, van Wijk JA, Lentze MJ*, et al.* (**2002**). Effect of corticosteroid therapy on serum cystatin C and beta2-microglobulin concentrations. Clin Chem 48**:** 1123-6.

103. White CA, Akbari A, Doucette S*, et al.* (**2009**). Effect of clinical variables and immunosuppression on serum cystatin C and beta-trace protein in kidney transplant recipients. Am J Kidney Dis 54**:** 922-30.
104. Cimerman N, Brguljan PM, Krasovec M*, et al.* (**2000**). Serum cystatin C, a potent inhibitor of cysteine proteinases, is elevated in asthmatic patients. Clin Chim Acta 300**:** 83-95.
105. Poge U, Gerhardt T, Bokenkamp A*, et al.* (**2004**). Time course of low molecular weight proteins in the early kidney transplantation period--influence of corticosteroids. Nephrol Dial Transplant 19**:** 2858-63.
106. Thervet E, Le Bricon T, Hugot V*, et al.* (**2000**). Early diagnosis of renal function recovery by cystatin C in renal allograft recipients. Transplant Proc 32**:** 2779.
107. Le Bricon T, Thervet E, Benlakehal M*, et al.* (**1999**). Changes in plasma cystatin C after renal transplantation and acute rejection in adults. Clin Chem 45**:** 2243-9.
108. Hall IE, Doshi MD, Poggio ED*, et al.* (**2011**). A comparison of alternative serum biomarkers with creatinine for predicting allograft function after kidney transplantation. Transplantation 91**:** 48-56.
109. Hall IE, Koyner JL, Doshi MD*, et al.* (**2011**). Urine cystatin C as a biomarker of proximal tubular function immediately after kidney transplantation. Am J Nephrol 33**:** 407-13.
110. Liu J. (**2012**). Evaluation of serum cystatin C for diagnosis of acute rejection after renal transplantation. Transplant Proc 44**:** 1250-3.
111. Christensson A, Ekberg J, Grubb A*, et al.* (**2003**). Serum cystatin C is a more sensitive and more accurate marker of glomerular filtration rate than enzymatic measurements of creatinine in renal transplantation. Nephron Physiol 94**:** p19-27.
112. Poge U, Gerhardt T, Stoffel-Wagner B*, et al.* (**2006**). Cystatin C-based calculation of glomerular filtration rate in kidney transplant recipients. Kidney Int 70**:** 204-10.
113. White C, Akbari A, Hussain N*, et al.* (**2007**). Chronic kidney disease stage in renal transplantation classification using cystatin C and creatinine-based equations. Nephrol Dial Transplant 22**:** 3013-20.
114. Maillard N, Mariat C, Bonneau C*, et al.* (**2008**). Cystatin C-based equations in renal transplantation: moving toward a better glomerular filtration rate prediction? Transplantation 85**:** 1855-8.
115. White C, Akbari A, Hussain N*, et al.* (**2005**). Estimating glomerular filtration rate in kidney transplantation: a comparison between serum creatinine and cystatin C-based methods. J Am Soc Nephrol 16**:** 3763-70.
116. Haase-Fielitz A, Haase M, Devarajan P. (**2014**). Neutrophil gelatinase-associated lipocalin as a biomarker of acute kidney injury: a critical evaluation of current status. Ann Clin Biochem 51**:** 335-51.
117. Haase-Fielitz A, Bellomo R, Devarajan P*, et al.* (**2009**). The predictive performance of plasma neutrophil gelatinase-associated lipocalin (NGAL) increases with grade of acute kidney injury. Nephrol Dial Transplant 24**:** 3349-54.

118. Haase M, Haase-Fielitz A, Bellomo R*, et al.* (**2011**). Neutrophil gelatinase-associated lipocalin as a marker of acute renal disease. Curr Opin Hematol 18**:** 11-8.
119. Haase M, Devarajan P, Haase-Fielitz A*, et al.* (**2011**). The outcome of neutrophil gelatinase-associated lipocalin-positive subclinical acute kidney injury: a multicenter pooled analysis of prospective studies. J Am Coll Cardiol 57**:** 1752-61.
120. Haase M, Bellomo R, Haase-Fielitz A. (**2011**). Neutrophil gelatinase-associated lipocalin: a superior biomarker for detection of subclinical acute kidney injury and poor prognosis. Biomark Med 5**:** 415-7.
121. Haase M, Bellomo R, Haase-Fielitz A. (**2010**). Neutrophil gelatinase-associated lipocalin. Curr Opin Crit Care 16**:** 526-32.
122. Haase M, Bellomo R, Devarajan P*, et al.* (**2009**). Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. Am J Kidney Dis 54**:** 1012-24.
123. Devarajan P. (**2010**). Neutrophil gelatinase-associated lipocalin: a promising biomarker for human acute kidney injury. Biomark Med 4**:** 265-80.
124. Devarajan P. (**2010**). Review: neutrophil gelatinase-associated lipocalin: a troponin-like biomarker for human acute kidney injury. Nephrology (Carlton) 15**:** 419-28.
125. Devarajan P. (**2008**). Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of kidney disease. Scand J Clin Lab Invest Suppl 241**:** 89-94.
126. Devarajan P. (**2008**). Neutrophil gelatinase-associated lipocalin--an emerging troponin for kidney injury. Nephrol Dial Transplant 23**:** 3737-43.
127. Devarajan P. (**2007**). Neutrophil gelatinase-associated lipocalin: new paths for an old shuttle. Cancer Ther 5**:** 463-70.
128. Schmidt-Ott KM, Mori K, Li JY*, et al.* (**2007**). Dual action of neutrophil gelatinase-associated lipocalin. J Am Soc Nephrol 18**:** 407-13.
129. Bagshaw SM, Bennett M, Haase M*, et al.* (**2010**). Plasma and urine neutrophil gelatinase-associated lipocalin in septic versus non-septic acute kidney injury in critical illness. Intensive Care Med 36**:** 452-61.
130. Kjeldsen L, Johnsen AH, Sengelov H*, et al.* (**1993**). Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. J Biol Chem 268**:** 10425-32.
131. Goetz DH, Willie ST, Armen RS*, et al.* (**2000**). Ligand preference inferred from the structure of neutrophil gelatinase associated lipocalin. Biochemistry 39**:** 1935-41.
132. Strong RK, Bratt T, Cowland JB*, et al.* (**1998**). Expression, purification, crystallization and crystallographic characterization of dimeric and monomeric human neutrophil gelatinase associated lipocalin (NGAL). Acta Crystallogr D Biol Crystallogr 54**:** 93-5.
133. Flower DR. (**2000**). Experimentally determined lipocalin structures. Biochim Biophys Acta 1482**:** 46-56.
134. Newcomer ME, Ong DE. (**2000**). Plasma retinol binding protein: structure and function of the prototypic lipocalin. Biochim Biophys Acta 1482**:** 57-64.

135. Weichsel A, Andersen JF, Champagne DE*, et al.* (**1998**). Crystal structures of a nitric oxide transport protein from a blood-sucking insect. Nat Struct Biol 5**:** 304-9.
136. Larsson J, Allhorn M, Kerstrom B. (**2004**). The lipocalin alpha(1)-microglobulin binds heme in different species. Arch Biochem Biophys 432**:** 196-204.
137. Goetz DH, Holmes MA, Borregaard N*, et al.* (**2002**). The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. Mol Cell 10**:** 1033-43.
138. Fjaertoft G, Foucard T, Xu S*, et al.* (**2005**). Human neutrophil lipocalin (HNL) as a diagnostic tool in children with acute infections: a study of the kinetics. Acta Paediatr 94**:** 661-6.
139. Schmidt-Ott KM, Mori K, Kalandadze A*, et al.* (**2006**). Neutrophil gelatinase-associated lipocalin-mediated iron traffic in kidney epithelia. Curr Opin Nephrol Hypertens 15**:** 442-9.
140. Cowland JB, Sorensen OE, Sehested M*, et al.* (**2003**). Neutrophil gelatinase-associated lipocalin is up-regulated in human epithelial cells by IL-1 beta, but not by TNF-alpha. J Immunol 171**:** 6630-9.
141. Liu Q, Nilsen-Hamilton M. (**1995**). Identification of a new acute phase protein. J Biol Chem 270**:** 22565-70.
142. Mallbris L, O'Brien KP, Hulthen A*, et al.* (**2002**). Neutrophil gelatinase-associated lipocalin is a marker for dysregulated keratinocyte differentiation in human skin. Exp Dermatol 11**:** 584-91.
143. Playford RJ, Belo A, Poulsom R*, et al.* (**2006**). Effects of mouse and human lipocalin homologues 24p3/lcn2 and neutrophil gelatinase-associated lipocalin on gastrointestinal mucosal integrity and repair. Gastroenterology 131**:** 809-17.
144. Ryon J, Bendickson L, Nilsen-Hamilton M. (**2002**). High expression in involuting reproductive tissues of uterocalin/24p3, a lipocalin and acute phase protein. Biochem J 367**:** 271-7.
145. Sorensen OE, Thapa DR, Roupe KM*, et al.* (**2006**). Injury-induced innate immune response in human skin mediated by transactivation of the epidermal growth factor receptor. J Clin Invest 116**:** 1878-85.
146. Nielsen BS, Borregaard N, Bundgaard JR*, et al.* (**1996**). Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. Gut 38**:** 414-20.
147. Stoesz SP, Friedl A, Haag JD*, et al.* (**1998**). Heterogeneous expression of the lipocalin NGAL in primary breast cancers. Int J Cancer 79**:** 565-72.
148. Monier F, Surla A, Guillot M*, et al.* (**2000**). Gelatinase isoforms in urine from bladder cancer patients. Clin Chim Acta 299**:** 11-23.
149. Cowland JB, Borregaard N. (**1997**). Molecular characterization and pattern of tissue expression of the gene for neutrophil gelatinase-associated lipocalin from humans. Genomics 45**:** 17-23.
150. Mori K, Lee HT, Rapoport D*, et al.* (**2005**). Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest 115**:** 610-21.
151. Mishra J, Dent C, Tarabishi R*, et al.* (**2005**). Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. Lancet 365**:** 1231-8.

152. Nickolas TL, O'Rourke MJ, Yang J*, et al.* (**2008**). Sensitivity and specificity of a single emergency department measurement of urinary neutrophil gelatinase-associated lipocalin for diagnosing acute kidney injury. Ann Intern Med 148**:** 810-9.
153. Nickolas TL, Schmidt-Ott KM, Canetta P*, et al.* (**2012**). Diagnostic and prognostic stratification in the emergency department using urinary biomarkers of nephron damage: a multicenter prospective cohort study. J Am Coll Cardiol 59**:** 246-55.
154. Parikh CR, Coca SG, Thiessen-Philbrook H*, et al.* (**2011**). Postoperative biomarkers predict acute kidney injury and poor outcomes after adult cardiac surgery. J Am Soc Nephrol 22**:** 1748-57.
155. Bennett M, Dent CL, Ma Q*, et al.* (**2008**). Urine NGAL predicts severity of acute kidney injury after cardiac surgery: a prospective study. Clin J Am Soc Nephrol 3**:** 665-73.
156. Cruz DN, de Cal M, Garzotto F*, et al.* (**2010**). Plasma neutrophil gelatinase-associated lipocalin is an early biomarker for acute kidney injury in an adult ICU population. Intensive Care Med 36**:** 444-51.
157. Wagener G, Jan M, Kim M*, et al.* (**2006**). Association between increases in urinary neutrophil gelatinase-associated lipocalin and acute renal dysfunction after adult cardiac surgery. Anesthesiology 105**:** 485-91.
158. de Geus HR, Bakker J, Lesaffre EM*, et al.* (**2011**). Neutrophil gelatinase-associated lipocalin at ICU admission predicts for acute kidney injury in adult patients. Am J Respir Crit Care Med 183**:** 907-14.
159. Grzyb J, Latowski D, Strzalka K. (**2006**). Lipocalins - a family portrait. J Plant Physiol 163**:** 895-915.
160. Mishra J, Ma Q, Prada A*, et al.* (**2003**). Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. J Am Soc Nephrol 14**:** 2534-43.
161. Amin RP, Vickers AE, Sistare F*, et al.* (**2004**). Identification of putative gene based markers of renal toxicity. Environ Health Perspect 112**:** 465-79.
162. Mishra J, Mori K, Ma Q*, et al.* (**2004**). Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity. Am J Nephrol 24**:** 307-15.
163. Schmidt-Ott KM. (**2011**). Neutrophil gelatinase-associated lipocalin as a biomarker of acute kidney injury--where do we stand today? Nephrol Dial Transplant 26**:** 762-4.
164. Parikh CR, Jani A, Mishra J*, et al.* (**2006**). Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. Am J Transplant 6**:** 1639-45.
165. Kusaka M, Kuroyanagi Y, Mori T*, et al.* (**2008**). Serum neutrophil gelatinase-associated lipocalin as a predictor of organ recovery from delayed graft function after kidney transplantation from donors after cardiac death. Cell Transplant 17**:** 129-34.
166. Magnusson NE, Hornum M, Jorgensen KA*, et al.* (**2012**). Plasma neutrophil gelatinase associated lipocalin (NGAL) is associated with kidney function in uraemic patients before and after kidney transplantation. BMC Nephrol 13**:** 8.

167. Kusaka M, Iwamatsu F, Kuroyanagi Y*, et al.* (**2012**). Serum neutrophil gelatinase associated lipocalin during the early postoperative period predicts the recovery of graft function after kidney transplantation from donors after cardiac death. J Urol 187**:** 2261-7.
168. Lebkowska U, Malyszko J, Lebkowska A*, et al.* (**2009**). Neutrophil gelatinase-associated lipocalin and cystatin C could predict renal outcome in patients undergoing kidney allograft transplantation: a prospective study. Transplant Proc 41**:** 154-7.
169. Mishra J, Ma Q, Kelly C*, et al.* (**2006**). Kidney NGAL is a novel early marker of acute injury following transplantation. Pediatr Nephrol 21**:** 856-63.
170. Lee EY, Kim MS, Park Y*, et al.* (**2012**). Serum neutrophil gelatinase-associated lipocalin and interleukin-18 as predictive biomarkers for delayed graft function after kidney transplantation. J Clin Lab Anal 26**:** 295-301.
171. Hall IE, Doshi MD, Reese PP*, et al.* (**2012**). Association between Peritransplant Kidney Injury Biomarkers and 1-Year Allograft Outcomes. Clin J Am Soc Nephrol 7**:** 1224-33.
172. Choi HM, Park KT, Lee JW*, et al.* (**2013**). Urine neutrophil gelatinase-associated lipocalin predicts graft outcome up to 1 year after kidney transplantation. Transplant Proc 45**:** 122-8.
173. Hollmen ME, Kyllönen LE, Inkinen KA*, et al.* (**2011**). Deceased donor neutrophil gelatinase-associated lipocalin and delayed graft function after kidney transplantation: a prospective study. Crit Care 15**:** R121.
174. Li C, Jackson RM. (**2002**). Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am J Physiol Cell Physiol 282**:** C227-41.
175. Eltzschig HK, Collard CD. (**2004**). Vascular ischaemia and reperfusion injury. Br Med Bull 70**:** 71-86.
176. Dolegowska B, Blogowski W, Domanski L. (**2012**). Association between the perioperative antioxidative ability of platelets and early post-transplant function of kidney allografts: a pilot study. PLoS One 7**:** e29779.
177. Paller MS. (**1992**). Free radical-mediated postischemic injury in renal transplantation. Ren Fail 14**:** 257-60.
178. Valko M, Izakovic M, Mazur M*, et al.* (**2004**). Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 266**:** 37-56.
179. Valko M, Rhodes CJ, Moncol J*, et al.* (**2006**). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160**:** 1-40.
180. Oberg BP, McMenamin E, Lucas FL*, et al.* (**2004**). Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. Kidney Int 65**:** 1009-16.
181. Mimic-Oka J, Simic T, Djukanovic L*, et al.* (**1999**). Alteration in plasma antioxidant capacity in various degrees of chronic renal failure. Clin Nephrol 51**:** 233-41.
182. Simic-Ogrizovic S, Simic T, Reljic Z*, et al.* (**1998**). Markers of oxidative stress after renal transplantation. Transpl Int 11 Suppl 1**:** S125-9.
183. Campise M, Bamonti F, Novembrino C*, et al.* (**2003**). Oxidative stress in kidney transplant patients. Transplantation 76**:** 1474-8.

184. Zahmatkesh M, Kadkhodaee M, Mahdavi-Mazdeh M*, et al.* (**2010**). Oxidative stress status in renal transplant recipients. Exp Clin Transplant 8**:** 38-44.
185. Ardalan MR, Estakhri R, Hajipour B*, et al.* (**2013**). Erythropoietin ameliorates oxidative stress and tissue injury following renal ischemia/reperfusion in rat kidney and lung. Med Princ Pract 22**:** 70-4.
186. Djamali A. (**2007**). Oxidative stress as a common pathway to chronic tubulointerstitial injury in kidney allografts. Am J Physiol Renal Physiol 293**:** F445-55.
187. Collard CD, Gelman S. (**2001**). Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. Anesthesiology 94**:** 1133-8.
188. Lee YJ, Park SH, Jeung TO*, et al.* (**2005**). Effect of adenosine triphosphate on phosphate uptake in renal proximal tubule cells: involvement of PKC and p38 MAPK. J Cell Physiol 205**:** 68-76.
189. Edelstein CL, Ling H, Schrier RW. (**1997**). The nature of renal cell injury. Kidney Int 51**:** 1341-51.
190. Dagher PC. (**2000**). Modeling ischemia in vitro: selective depletion of adenine and guanine nucleotide pools. Am J Physiol Cell Physiol 279**:** C1270-7.
191. Khand FD, Gordge MP, Robertson WG*, et al.* (**2002**). Mitochondrial superoxide production during oxalate-mediated oxidative stress in renal epithelial cells. Free Radic Biol Med 32**:** 1339-50.
192. Kim YK, Lee SK, Ha MS*, et al.* (**2002**). Differential role of reactive oxygen species in chemical hypoxia-induced cell injury in opossum kidney cells and rabbit renal cortical slices. Exp Nephrol 10**:** 275-84.
193. Maenpaa CJ, Shames BD, Van Why SK*, et al.* (**2008**). Oxidant-mediated apoptosis in proximal tubular epithelial cells following ATP depletion and recovery. Free Radic Biol Med 44**:** 518-26.
194. Nilakantan V, Liang H, Maenpaa CJ*, et al.* (**2008**). Differential patterns of peroxynitrite mediated apoptosis in proximal tubular epithelial cells following ATP depletion recovery. Apoptosis 13**:** 621-33.
195. Ponticelli C. (**2013**). Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. Nephrol Dial Transplant.
196. Hariharan N, Zhai P, Sadoshima J. (**2011**). Oxidative stress stimulates autophagic flux during ischemia/reperfusion. Antioxid Redox Signal 14**:** 2179-90.
197. Waller HL, Harper SJ, Hosgood SA*, et al.* (**2006**). Biomarkers of oxidative damage to predict ischaemia-reperfusion injury in an isolated organ perfusion model of the transplanted kidney. Free Radic Res 40**:** 1218-25.
198. Deneke SM, Fanburg BL. (**1989**). Regulation of cellular glutathione. Am J Physiol 257**:** L163-73.
199. Weinberg JM. (**1992**). Glutathione and glycine in acute renal failure. Ren Fail 14**:** 311-9.
200. McCord JM. (**1985**). Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 312**:** 159-63.

201. Granger DN. (**1988**). Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. Am J Physiol 255**:** H1269-75.
202. Parajuli N, MacMillan-Crow LA. (**2013**). Role of reduced manganese superoxide dismutase in ischemia-reperfusion injury: a possible trigger for autophagy and mitochondrial biogenesis? Am J Physiol Renal Physiol 304**:** F257-67.
203. Nilakantan V, Halligan NL, Nguyen TK*, et al.* (**2005**). Post-translational modification of manganese superoxide dismutase in acutely rejecting cardiac transplants: role of inducible nitric oxide synthase. J Heart Lung Transplant 24**:** 1591-9.
204. Nilakantan V, Zhou X, Hilton G*, et al.* (**2005**). Hierarchical change in antioxidant enzyme gene expression and activity in acute cardiac rejection: role of inducible nitric oxide synthase. Mol Cell Biochem 270**:** 39-47.
205. Whitin JC, Tham DM, Bhamre S*, et al.* (**1998**). Plasma glutathione peroxidase and its relationship to renal proximal tubule function. Mol Genet Metab 65**:** 238-45.
206. Zachara BA, Wlodarczyk Z, Masztalerz M*, et al.* (**2004**). Selenium concentrations and glutathione peroxidase activities in blood of patients before and after allogenic kidney transplantation. Biol Trace Elem Res 97**:** 1-13.
207. Vostalova J, Galandakova A, Svobodova AR*, et al.* (**2012**). Time-course evaluation of oxidative stress-related biomarkers after renal transplantation. Ren Fail 34**:** 413-9.
208. Djamali A, Reese S, Yracheta J*, et al.* (**2005**). Epithelial-to-mesenchymal transition and oxidative stress in chronic allograft nephropathy. Am J Transplant 5**:** 500-9.
209. Sahin-Efe A, Katsikeris F, Mantzoros CS. (**2012**). Advances in adipokines. Metabolism 61**:** 1659-65.
210. Wannamethee SG, Tchernova J, Whincup P*, et al.* (**2007**). Plasma leptin: associations with metabolic, inflammatory and haemostatic risk factors for cardiovascular disease. Atherosclerosis 191**:** 418-26.
211. Nakamura K, Fuster JJ, Walsh K. (**2014**). Adipokines: a link between obesity and cardiovascular disease. J Cardiol 63**:** 250-9.
212. Van de Voorde J, Pauwels B, Boydens C*, et al.* (**2013**). Adipocytokines in relation to cardiovascular disease. Metabolism 62**:** 1513-21.
213. Becker B, Kronenberg F, Kielstein JT*, et al.* (**2005**). Renal insulin resistance syndrome, adiponectin and cardiovascular events in patients with kidney disease: the mild and moderate kidney disease study. J Am Soc Nephrol 16**:** 1091-8.
214. Cumin F, Baum HP, Levens N. (**1996**). Leptin is cleared from the circulation primarily by the kidney. Int J Obes Relat Metab Disord 20**:** 1120-6.
215. Kataoka H, Sharma K. (**2006**). Renal handling of adipokines. Contrib Nephrol 151**:** 91-105.
216. Komura N, Kihara S, Sonoda M*, et al.* (**2010**). Increment and impairment of adiponectin in renal failure. Cardiovasc Res 86**:** 471-7.
217. Moraes-Vieira PM, Bassi EJ, Araujo RC*, et al.* (**2012**). Leptin as a link between the immune system and kidney-related diseases: leading actor or just a coadjuvant? Obes Rev 13**:** 733-43.

218. Saraheimo M, Forsblom C, Fagerudd J*, et al.* (**2005**). Serum adiponectin is increased in type 1 diabetic patients with nephropathy. Diabetes Care 28**:** 1410-4.
219. Shoji T, Nishizawa Y, Emoto M*, et al.* (**1997**). Renal function and insulin resistance as determinants of plasma leptin levels in patients with NIDDM. Diabetologia 40**:** 676-9.
220. Zoccali C, Mallamaci F, Tripepi G. (**2003**). Adipose tissue as a source of inflammatory cytokines in health and disease: focus on end-stage renal disease. Kidney Int Suppl**:** S65-8.
221. Ziegelmeier M, Bachmann A, Seeger J*, et al.* (**2008**). Adipokines influencing metabolic and cardiovascular disease are differentially regulated in maintenance hemodialysis. Metabolism 57**:** 1414-21.
222. Roubicek T, Bartlova M, Krajickova J*, et al.* (**2009**). Increased production of proinflammatory cytokines in adipose tissue of patients with end-stage renal disease. Nutrition 25**:** 762-8.
223. Halaas JL, Gajiwala KS, Maffei M*, et al.* (**1995**). Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269**:** 543-6.
224. Campfield LA, Smith FJ, Burn P. (**1996**). The OB protein (leptin) pathway--a link between adipose tissue mass and central neural networks. Horm Metab Res 28**:** 619-32.
225. Meyer C, Robson D, Rackovsky N*, et al.* (**1997**). Role of the kidney in human leptin metabolism. Am J Physiol 273**:** E903-7.
226. Cumin F, Baum HP, Levens N. (**1997**). Mechanism of leptin removal from the circulation by the kidney. J Endocrinol 155**:** 577-85.
227. Zeng J, Patterson BW, Klein S*, et al.* (**1997**). Whole body leptin kinetics and renal metabolism in vivo. Am J Physiol 273**:** E1102-6.
228. Zoccali C, Mallamaci F, Panuccio V*, et al.* (**2003**). Adiponectin is markedly increased in patients with nephrotic syndrome and is related to metabolic risk factors. Kidney Int Suppl**:** S98-102.
229. Frystyk J, Tarnow L, Hansen TK*, et al.* (**2005**). Increased serum adiponectin levels in type 1 diabetic patients with microvascular complications. Diabetologia 48**:** 1911-8.
230. Chudek J, Adamczak M, Karkoszka H*, et al.* (**2003**). Plasma adiponectin concentration before and after successful kidney transplantation. Transplant Proc 35**:** 2186-9.
231. Kokot F, Adamczak M, Wiecek A. (**1998**). Plasma leptin concentration in kidney transplant patients during the early post-transplant period. Nephrol Dial Transplant 13**:** 2276-80.
232. Kokot F, Adamczak M, Wiecedil*, et al.* (**1999**). Plasma immunoreactive leptin and neuropeptide Y levels in kidney transplant patients. Am J Nephrol 19**:** 28-33.
233. El Haggan W, Chauveau P, Barthe N*, et al.* (**2004**). Serum leptin, body fat, and nutritional markers during the six months post-kidney transplantation. Metabolism 53**:** 614-9.
234. Souza GC, Costa C, Scalco R*, et al.* (**2008**). Serum leptin, insulin resistance, and body fat after renal transplantation. J Ren Nutr 18**:** 479-88.
235. Landt M, Brennan DC, Parvin CA*, et al.* (**1998**). Hyperleptinaemia of end-stage renal disease is corrected by renal transplantation. Nephrol Dial Transplant 13**:** 2271-5.
236. Howard JK, Lord GM, Clutterbuck EJ*, et al.* (**1997**). Plasma immunoreactive leptin concentration in end-stage renal disease. Clin Sci (Lond) 93**:** 119-26.

237. Beauchamp C, Fridovich I. (**1971**). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44**:** 276-87.
238. Shek DT, Ma CM. (**2011**). Longitudinal data analyses using linear mixed models in SPSS: concepts, procedures and illustrations. ScientificWorldJournal 11**:** 42-76.
239. G.M. F, M. D, G. V*, et al.* (**2008**). *Longitudinal Data Analysis: A Handbook of Modern Statistical Methods.* . London: Chapman & Hall/CRC Press.
240. Hedeker D, Mermelstein RJ, Demirtas H. (**2008**). An application of a mixed-effects location scale model for analysis of Ecological Momentary Assessment (EMA) data. Biometrics 64**:** 627-34.
241. DeLucia C, Pitts SC. (**2006**). Applications of individual growth curve modeling for pediatric psychology research. J Pediatr Psychol 31**:** 1002-23.
242. Gibbons RD, Hedeker D, Elkin I*, et al.* (**1993**). Some conceptual and statistical issues in analysis of longitudinal psychiatric data. Application to the NIMH treatment of Depression Collaborative Research Program dataset. Arch Gen Psychiatry 50**:** 739-50.
243. Laird NM, Ware JH. (**1982**). Random-effects models for longitudinal data. Biometrics 38**:** 963-74.
244. Bryk A, Raudenbush SW. (**1992**). *Hierarchical Linear Models for Social and Behavioral Research: Applications and Data Analysis Methods.* : Newbury Park, CA: Sage.
245. Bryk AS, Raudenbush SW. (**1987**). Application of hierarchical linear models to assessing change. . Psychological Bulletin 101**:** 147-58.
246. Snijders TA, Bosker RJ. (**2012**). *Multilevel Analysis: An Introduction to Basic and Advanced Multilevel Modeling*, second edition edn. Sage Publishers: London.
247. Fitzmaurice GM, Ravichandran C. (**2008**). A primer in longitudinal data analysis. Circulation 118**:** 2005-10.
248. Winter B. (**2013**). Linear models and linear mixed effects models in R with linguistic applications.
249. Latra N, Linuwih S., Purhadi;*, et al.* (**2010**). Estimation for Multivariate Linear Mixed Models. International Journal of Basic & Applied Sciences 10**:** 48-53.
250. Daniels MJ, Kass RE. (**2001**). Shrinkage estimators for covariance matrices. Biometrics 57**:** 1173-84.
251. Willett JB. (**1998**). Questions and answers in the measurement of change. Rev Res Educ 15**:** 345–422.
252. Shek DT. (**1998**). A longitudinal study of the relations of family factors to adolescent psychological symptoms, coping resources, school behavior, and substance abuse. Int J Adolesc Med Health 10**:** 155-84.
253. Kowalchuk RK, Keselman HJ, Algina J*, et al.* (**2004**). The analysis of repeated measurements with mixed-model adjusted F tests. Educ Psychol Meas 64(2)**:** 224–42.
254. Bono R, Arnau J, Balluerka N. (**2007**). Using linear mixed models in longitudinal studies: application of SAS PROC MIXED. Rev Elec Metodol Apl 12(2)**:** 15–31.

255. Littell RC, Pendergast J, Natarajan R. (**2000**). Modelling covariance structure in the analysis of repeated measures data. Stat Med 19**:** 1793-819.
256. Collett D. (**1994**). *Modelling Survival Data in Medical Research*. Chapman & Hall: London.
257. Kleinbaum DG, Klein M. (**2011**). *Survival Analysis- A Self-Learning Text*, Third Edition edn. Springer Publishers New York, Inc.
258. Pintilie M. (**2006**). *Competing risks - A practical perspective*. John Wiley & Sons, Lda.
259. Pintilie M. (**2007**). Analysing and interpreting competing risk data. Stat Med 26**:** 1360-7.
260. Gichangi A, Vach W. (**2005**). The analysis of competing risks data: a guided tour. . Statistics in Medicine 132**:** 1-41.
261. Grunkemeier GL, Jin R, Eijkemans MJ*, et al.* (**2007**). Actual and actuarial probabilities of competing risks: apples and lemons. Ann Thorac Surg 83**:** 1586-92.
262. Moeschberger ML, Tordoff KP, Kochar N. (**2008**). A Review of Statistical Analyses for Competing Risks. *Handbook of Statistics: Epidemiology and Medical Statistics*. Elsevier, pp 321-41.
263. Tsiatis A. (**1975**). A nonidentifiability aspect of the problem of competing risks. Proc Natl Acad Sci USA 72**:** 20-2.
264. Gooley TA, Leisenring W, Crowley J*, et al.* (**1999**). Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. Stat Med 18**:** 695-706.
265. Fine JP, Gray RJ. (**1999**). A proportional hazards model for the subdistribution of a competing risks. J Am Stat Assoc 94**:** 496-509.
266. Putter H, Fiocco M, Geskus RB. (**2007**). Tutorial in biostatistics: competing risks and multi-state models. Stat Med 26**:** 2389-430.
267. Prentice RL, Kalbfleisch JD, Peterson AV, Jr.*, et al.* (**1978**). The analysis of failure times in the presence of competing risks. Biometrics 34**:** 541-54.
268. Satagopan JM, Ben-Porat L, Berwick M*, et al.* (**2004**). A note on competing risks in survival data analysis. Br J Cancer 91**:** 1229-35.
269. Gjertson DW, Dabrowska DM, Cui X*, et al.* (**2002**). Four causes of cadaveric kidney transplant failure: a competing risk analysis. Am J Transplant 2**:** 84-93.
270. Noordzij M, Leffondre K, van Stralen KJ*, et al.* (**2013**). When do we need competing risks methods for survival analysis in nephrology? Nephrol Dial Transplant 28**:** 2670-7.
271. Oakes D, Peterson DR. (**2008**). Survival methods: additional topics. Circulation 117**:** 2949-55.
272. Koller MT, Raatz H, Steyerberg EW*, et al.* (**2012**). Competing risks and the clinical community: irrelevance or ignorance? Stat Med 31**:** 1089-97.
273. Giral-Classe M, Hourmant M, Cantarovich D*, et al.* (**1998**). Delayed graft function of more than six days strongly decreases long-term survival of transplanted kidneys. Kidney Int 54**:** 972-8.
274. Marcen R, Orofino L, Pascual J*, et al.* (**1998**). Delayed graft function does not reduce the survival of renal transplant allografts. Transplantation 66**:** 461-6.
275. Troppmann C, Gillingham KJ, Gruessner RW*, et al.* (**1996**). Delayed graft function in the absence of rejection has no long-term impact. A study of cadaver kidney recipients with good graft function at 1 year after transplantation. Transplantation 61**:** 1331-7.

276. Haynes R, Staplin N, Emberson J*, et al.* (**2014**). Evaluating the Contribution of the Cause of Kidney Disease to Prognosis in CKD: Results From the Study of Heart and Renal Protection (SHARP). Am J Kidney Dis 64**:** 40-8.
277. Watson CJ, Dark JH. (**2012**). Organ transplantation: historical perspective and current practice. Br J Anaesth 108 Suppl 1**:** i29-42.
278. Lodhi SA, Meier-Kriesche HU. (**2011**). Kidney allograft survival: the long and short of it. Nephrol Dial Transplant 26**:** 15-7.
279. Gill JS, Tonelli M, Mix CH*, et al.* (**2003**). The change in allograft function among long-term kidney transplant recipients. J Am Soc Nephrol 14**:** 1636-42.
280. Sijpkens YW, Bruijn JA, Paul LC. (**2001**). Chronic allograft nephropathy categorised in chronic interstitial and vascular rejection. Transplant Proc 33**:** 1153.
281. Almond PS, Troppmann C, Escobar F*, et al.* (**1991**). Economic impact of delayed graft function. Transplant Proc 23**:** 1304.
282. Rosenthal JT, Danovitch GM, Wilkinson A*, et al.* (**1991**). The high cost of delayed graft function in cadaveric renal transplantation. Transplantation 51**:** 1115-8.
283. Ojo AO, Hanson JA, Wolfe RA*, et al.* (**2000**). Long-term survival in renal transplant recipients with graft function. Kidney Int 57**:** 307-13.
284. Perez Fontan M, Rodriquez-Carmona A, Bouza P*, et al.* (**1996**). Outcome of grafts with long-lasting delayed function after renal transplantation. Transplantation 62**:** 42-7.
285. Boom H, Mallat MJ, de Fijter JW*, et al.* (**2000**). Delayed graft function influences renal function, but not survival. Kidney Int 58**:** 859-66.
286. Andersen PK, Geskus RB, de Witte T*, et al.* (**2012**). Competing risks in epidemiology: possibilities and pitfalls. Int J Epidemiol 41**:** 861-70.
287. Wolbers M, Koller MT, Witteman JC*, et al.* (**2009**). Prognostic models with competing risks: methods and application to coronary risk prediction. Epidemiology 20**:** 555-61.
288. Lau B, Cole SR, Gange SJ. (**2009**). Competing risk regression models for epidemiologic data. Am J Epidemiol 170**:** 244-56.
289. Yarlagadda SG, Coca SG, Garg AX*, et al.* (**2008**). Marked variation in the definition and diagnosis of delayed graft function: a systematic review. Nephrol Dial Transplant 23**:** 2995-3003.
290. Mallon DH, Summers DM, Bradley JA*, et al.* (**2013**). Defining delayed graft function after renal transplantation: simplest is best. Transplantation 96**:** 885-9.
291. Boom H, Paul L, Fijter J. (**2004**). Delayed graft function in renal transplantation. Transplantation Reviews 18**:** 139-52.
292. Hariharan S, Kasiske B, Matas A*, et al.* (**2004**). Surrogate markers for long-term renal allograft survival. Am J Transplant 4**:** 1179-83.
293. Hariharan S, McBride MA, Cherikh WS*, et al.* (**2002**). Post-transplant renal function in the first year predicts long-term kidney transplant survival. Kidney Int 62**:** 311-8.
294. Hariharan S, McBride MA, Cohen EP. (**2003**). Evolution of endpoints for renal transplant outcome. Am J Transplant 3**:** 933-41.

295. Cecka JM. (**1998**). The UNOS Scientific Renal Transplant Registry. Clin Transpl**:** 1-16.
296. Cecka M. (**1998**). Clinical outcome of renal transplantation. Factors influencing patient and graft survival. Surg Clin North Am 78**:** 133-48.
297. Govani MV, Kwon O, Batiuk TD*, et al.* (**2002**). Creatinine reduction ratio and 24-hour creatinine excretion on posttransplant day two: simple and objective tools to define graft function. J Am Soc Nephrol 13**:** 1645-9.
298. Kasiske BL, Andany MA, Danielson B. (**2002**). A thirty percent chronic decline in inverse serum creatinine is an excellent predictor of late renal allograft failure. Am J Kidney Dis 39**:** 762-8.
299. Meier-Kriesche HU, Baliga R, Kaplan B. (**2003**). Decreased renal function is a strong risk factor for cardiovascular death after renal transplantation. Transplantation 75**:** 1291-5.
300. Pascual J, Marcen R, Zamora J*, et al.* (**2009**). Very early serum creatinine as a surrogate marker for graft survival beyond 10 years. J Nephrol 22**:** 90-8.
301. Dominguez J, Kompatzki A, Norambuena R*, et al.* (**2005**). Benefits of early biopsy on the outcome of kidney transplantation. Transplant Proc 37**:** 3361-3.
302. Kaplan B, Schold J, Meier-Kriesche HU. (**2003**). Poor predictive value of serum creatinine for renal allograft loss. Am J Transplant 3**:** 1560-5.
303. Kaplan B, Meier-Kriesche HU. (**2002**). Death after graft loss: an important late study endpoint in kidney transplantation. Am J Transplant 2**:** 970-4.
304. Kuwabara T, Mori K, Mukoyama M*, et al.* (**2009**). Urinary neutrophil gelatinase-associated lipocalin levels reflect damage to glomeruli, proximal tubules, and distal nephrons. Kidney Int 75**:** 285-94.
305. Haase M, Bellomo R, Devarajan P*, et al.* (**2009**). Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. Am J Kidney Dis 54**:** 1012-24.
306. Maury E, Brichard SM. (**2010**). Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol Cell Endocrinol 314**:** 1-16.
307. Chachkhiani I, Gurlich R, Maruna P*, et al.* (**2005**). The postoperative stress response and its reflection in cytokine network and leptin plasma levels. Physiol Res 54**:** 279-85.
308. Hernandez C, Simo R, Chacon P*, et al.* (**2000**). Influence of surgical stress and parenteral nutrition on serum leptin concentration. Clin Nutr 19**:** 61-4.
309. Eltzschig HK, Eckle T. (**2011**). Ischemia and reperfusion--from mechanism to translation. Nat Med 17**:** 1391-401.
310. Buettner R, Bollheimer LC, Zietz B*, et al.* (**2002**). Definition and characterization of relative hypo- and hyperleptinemia in a large Caucasian population. J Endocrinol 175**:** 745-56.
311. Song Y, Yu Q, Zhang J*, et al.* (**2014**). Increased myocardial ischemia-reperfusion injury in renal failure involves cardiac adiponectin signal deficiency. Am J Physiol Endocrinol Metab 306**:** E1055-64.

312. Del Rio D, Stewart AJ, Pellegrini N. (**2005**). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis 15**:** 316-28.
313. Ayala A, Munoz MF, Arguelles S. (**2014**). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev 2014**:** 360438.
314. Ma MC, Chen YS, Huang HS. (**2014**). Erythrocyte oxidative stress in patients with calcium oxalate stones correlates with stone size and renal tubular damage. Urology 83**:** 510 e9-17.
315. Pande D, Negi R, Karki K*, et al.* (**2012**). Oxidative damage markers as possible discriminatory biomarkers in breast carcinoma. Transl Res 160**:** 411-8.
316. Polidori MC, Savino K, Alunni G*, et al.* (**2002**). Plasma lipophilic antioxidants and malondialdehyde in congestive heart failure patients: relationship to disease severity. Free Radic Biol Med 32**:** 148-52.
317. Romero MJ, Bosch-Morell F, Romero B*, et al.* (**1998**). Serum malondialdehyde: possible use for the clinical management of chronic hepatitis C patients. Free Radic Biol Med 25**:** 993-7.
318. Kim J, Seok YM, Jung KJ*, et al.* (**2009**). Reactive oxygen species/oxidative stress contributes to progression of kidney fibrosis following transient ischemic injury in mice. Am J Physiol Renal Physiol 297**:** F461-70.
319. Fonseca I, Reguengo H, Almeida M*, et al.* (**2014**). Oxidative stress in kidney transplantation: malondialdehyde is an early predictive marker of graft dysfunction. Transplantation 97**:** 1058-65.
320. Cristol JP, Vela C, Maggi MF*, et al.* (**1998**). Oxidative stress and lipid abnormalities in renal transplant recipients with or without chronic rejection. Transplantation 65**:** 1322-8.
321. Domanski L, Dolegowska B, Safranow K*, et al.* (**2006**). Activity of CuZn-superoxide dismutase, catalase and glutathione peroxidase in erythrocytes in kidney allografts during reperfusion in patients with and without delayed graft function. Clin Transplant 20**:** 67-71.

ERRATA for PhD Thesis "*Predictive factors of graft dysfunction and long-term kidney allograft failure*" - Isabel Fonseca, May 21, 2015

- **Page 75**. Paper III, Fig. 1, after the ROC curves graph and before the legend, the table with the biomarkers AUCs was not printed in the paper.

	AUC (95% CI)	***P*-Value**
Day 1 (8-to-12 h after surgery)		
MDA (µmol/L)	0.90 (0.81 - 0.99)	< 0.001
Serum Creatinine (mg/dl)	0.73 (0.58 - 0.89)	0.012
Serum Cystatin C (mg/L)	0.91 (0.82 - 1.00)	< 0.001
Change from pre-transplant to first day after KTx		
MDA (µmol/L)	0.84 (0.70 - 0.97)	< 0.001
Serum Creatinine (mg/dl)	0.69 (0.52 - 0.87)	0.036