# The impact of cerebral injury in donation and transplantation

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# Chapter 6 **Donor Serum Angiopoietin 2 is an Independent Predictor of Kidney Transplant Outcome**

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## Abstract

Background: Donor-derived biomarkers that have predictive value for posttransplant outcome are useful to prevent unnecessary discard of donor organs and to fine-tune post-operative treatment of the recipient. Recently, we found that serum angiopoietin levels are elevated in brain dead donors. Angiopoietin-2 is a prognostic survival marker in critically ill patients. In this study we investigated whether donor angiopoietins have a predictive value in recipient renal transplantation.

Methods: From 297 deceased kidney donors included in an international prospective randomized controlled trial, serum was analyzed for angiopoietin-1 and angiopoietin-2. Using multivariate models we tested whether donor serum angiopoietins were independently associated with delayed graft function (DGF), primary non-function (PNF) and graft survival (GS).

Results: Serum angiopoietin-2 concentration was significantly associated with GS: higher values in donor serum were predictive of a lower risk of graft failure (HR=0.91, p=0.027). For angiopoietin-1 no association with GS could be found. Donor angiopoietin levels had no predictive value for DGF and PNF.

Conclustion: The present study shows that angiopoietin-2 measured in donor serum prior to donation is an independent predictor of kidney graft survival.

## Introduction

Kidneys derived from living donors show a better short and long term posttransplant performance compared to renal grafts recovered from deceased donors (1;2).To date, the majority of organs is still derived from deceased donors (3;4). Various donor-related factors are known to have a relevant impact on graft outcome in the recipient, including donor history of hypertension and diabetes mellitus, cause of death, donor type, age, sex, race, warm and cold ischemic time, and the type of organ preservation (5;6). In an attempt to predict deceased donor graft quality, many American centers routinely utilize pre-transplant donor biopsies. However, the prognostic value of these biopsies remains uncertain (7). Recently, Murugan et al. published results of a study which showed that an increased plasma interleukin-6 concentration in the donor is associated with lower recipient hospital-free survival after transplantation (8). Apart from these findings, to date no donor biomarkers are available that have a relevant and independent predictive value for kidney transplant outcome.

In the past years, we and other groups have found a progressive expression of pro-inflammatory and pro-coagulatory markers after induction of brain death in animal models and human donors (9). Brain death and the upregulation of the innate immune response has been associated with decreased organ viability and a higher risk inferior outcome after transplantation (1;2;10;11).

Angiopoietin-1 (ang-1) and angiopoietin-2 (ang-2) are both antagonistic regulatory proteins which play an important role in vascular inflammation. The angiopoietin–Tie ligand-receptor system is crucial in regulating vascular integrity and quiescence (12). Ang-1 dampens the inflammatory response while ang-2 boosts this response (13). An imbalance of this factors predisposes for pre-eclampsia and survival in critically ill and trauma patients (14-16). Furthermore, it has been shown that in the presence of sepsis, higher circulating ang-2 levels are associated with an increased mortality (16).

Recently, we found that in a small group of 30 donors after brain death donation (DBD), an increased pro-inflammatory ang-2 response was present compared to living donors. Higher ang-1 levels were observed just after the diagnosis of brain stem death in DBD donors (17). Based on literature and on the findings of our previous study, we hypothesized that ang-1 and ang-2 levels measured in kidney donors might be promising biomarkers to predict renal transplant outcome.

In the present study, we assessed outcomes of 297 kidney transplant recipients included in an international prospective randomized controlled trial on machine preservation vs. cold storage (18). The concentrations of ang-1 and ang-2 were measured in donor serum and these values were correlated with posttransplant outcome in the recipient. The aim of this study was to assess the potential of donor serum ang-1 and ang-2 as a biomarker to predict renal transplant success.

# Methods

## STUDY DESIGN

The present study is a sub-study of the investigator-driven international randomized controlled trial which investigated the effect of hypothermic machine perfusion versus static cold storage preservation and included the Netherlands, Belgium, and the federal state of North Rhine-Westphalia in Germany (The Machine Preservation Trial). Between November 1, 2005 and August 17, 2007, all consecutive deceased donor kidney pairs that met the initial inclusion criteria were eligible for randomization by Eurotransplant, an international organ exchange organization in Europe. Both, donation after brain death (DBD) and donation after cardiac death (DCD) donors were included. From each donor, one kidney was randomly assigned to machine perfusion and the contralateral kidney to cold storage. The organs could be transplanted into any recipient within the Eurotransplant region (4). For further details on study design, inclusion criteria, and recipient follow up we refer to our previous publication(18).

## SAMPLE COLLECTION

Whole-blood samples of 8 ml were drawn prior to organ recovery in the donor. In DCD procedures the sample was taken just before withdrawal of treatment. In brain dead donors, blood was drawn in the operating room at the start of procurement. Samples were transported on ice, centrifuged to obtain serum, and then stored at -80°C until further analysis.

## SERUM ANGIOPOIETIN 1 AND 2 ANALYSIS

Human ang-1 and ang-2 enzyme-linked immunoassay (ELISA) test kits (R&D systems, Minneapolis, USA) were used according to the manufacturer's instructions, to evaluate ang-1 and ang-2 in donor serum samples. All samples were tested in duplicate and analyzed at 450 nm using a micro-plate spectrophotometer (Victor3, 1420 multi-label counter, Perkin Elmer).

## STUDY END POINTS

The end point to assess short term graft performance was delayed graft function (DGF), defined as the requirement for dialysis during the first week after transplantation. The other end points were primary non-function (PNF), and death censored graft survival up to 1 year after transplantation.

## STATISTICAL ANALYSIS

First, univariate analyses were conducted to investigate the association between donor serum ang-1 or ang-2 levels and the end points for posttransplant outcome. We used the Mann Whitney test to compare angiopoietin concentrations between recipients with and without delayed graft function. A similar analysis was performed for the primary non-function end point. Kaplan-Meier survival curves and logrank tests were used to assess whether 1 year death censored graft survival was significantly different in recipients whose kidney donor had an ang-1 or ang-2

concentration above the median, versus patients whose donor had an ang-1 or ang-2 level under the median.

For those univariate associations that were found to be statistically significant, a multivariate model was built. In the present study, this was only necessary for the association between ang-2 and graft survival, but for the sake of completeness we decided to also perform a multivariate analysis for the influence of ang-1 on graft survival. Cox proportional hazards models were constructed to test whether donor serum ang-1 or ang-2 concentrations were significant independent predictors for the risk of graft failure in the first year posttransplant. Apart from ang-1 or ang-2 concentration, other covariates in these models were those listed in Table 2. In addition, we incorporated a normal gamma frailty term for the donor into each Cox model, to account for the within-donor dependence structure of our data (for each left + right kidney in a pair donor characteristics were by definition exactly the same, but recipients were different) (19).

Statistical analyses were conducted using SPSS (version 16.0) and R (version 2.7.1) software packages. Two-sided p-values under 0.05 were considered to indicate statistical significance.

# Results

## DONORS, RECIPIENTS, AND SERUM SAMPLES

Between November 1, 2005 and August 17, 2007, 297 deceased kidney donors 16 years of age or older were included in this sub-study. In the original study a total of 376 donors were included. Seventy-nine donors were excluded, since no donor serum samples were available. Characteristics of the 297 donors (and 594 recipients) with stored serum samples are shown in table 1. Baseline characteristics of these donors and recipients included in the present study did not differ significantly from those in the original group.

## ANGIOPOIETIN 1 AND 2 LEVELS

Serum levels of ang-1 and ang-2 were elevated in most deceased donors. The upper reference value in healthy individuals for ang-1 is 6 ng/ml and for ang-2 2.5 ng/ml (17;20). The median (+ interquartile range) of ang-1 was 16.5 (9.4–25.8), and of ang-2 was 4.7 (2.8–8.4). The distribution of ang-1 and ang-2 values is shown in figure 1. As plotted in figure 2a-f, both ang-1 and ang-2 levels, this figures suggests no difference between the sub-groups of DCD donors vs. DBD donors, delayed vs. immediately functioning grafts, and grafts with primary non-function (PNF) vs. kidneys without PNF.

**Table 1: Donor, recipient, and transplant demographics and overall posttransplant outcome.**

| | Whole group (N = 752 recipients) | Subgroup[a] (N = 594 recipients) | P value[b] |
|---|---|---|---|
| Donor demographics | | | |
| Donor age[c] (yr) | 50 (16-81) | 50 (17-81) | 0.8 |
| Female donor (%) | 42 | 41 | 0.9 |
| DCD donor (%) | 22 | 20 | 0.4 |
| ECD donor (%) | 28 | 26 | 0.5 |
| Traumatic cause of death (%) | 23 | 23 | 0.9 |
| Donor history of hypertension (%) | 23 | 21 | 0.6 |
| Donor history of diabetes mellitus (%) | 5 | 4 | 1.0 |
| Recipient demographics | | | |
| Recipient age[c] (yr) | 53 (2–79) | 52 (8–79) | 0.3 |
| Female recipient (%) | 41 | 41 | 0.9 |
| Total time spent on the waiting list[c] (yr) | 5 (1–8) | 5 (1–8) | 0.9 |
| Previous transplants (% ≥1) | 30 | 28 | 0.5 |
| PRA level >5% (%) | 11 | 11 | 0.7 |
| Immunosuppressive drugs (%) | | | |
| Prednisolone | 98 | 98 | 0.8 |
| Cyclosporine | 49 | 50 | 1.0 |
| Tacrolimus | 50 | 49 | 1.0 |
| Azathioprine | 1 | 1 | 1.0 |
| Mycophenolate mofetil | 86 | 87 | 0.7 |
| Antithymocyte globulin | 14 | 14 | 0.8 |
| Transplant demographics | | | |
| HLA mismatches (% of 0 mismatches) | 15 | 16 | 0.7 |
| Cold ischemic time[c] (h) | 15 (2–47) | 15 (2–47) | 0.4 |
| Organ preservation method[d] (%) | | | |
| Static cold storage | 50 | 50 | - |
| Hypothermic machine perfusion | 50 | 50 | - |
| Posttransplant outcome | | | |
| Delayed graft function (%) | 28 | 27 | 0.7 |
| Duration of delayed graft function (days)[c] | 13 (1–93) | 13 (1–35) | 0.5 |
| Primary non-function (%) | 3.3 | 3.4 | 1.0 |
| Any acute rejection in first year (%) | 24 | 25 | 0.8 |
| 1 year death censored graft survival (%) | 92 | 93 | 0.8 |

**aThe subgroup of recipients for whom a donor serum sample was available to analyze angiopoietin 1 and 2. bAll p values are two-sided. Mann-Whitney test for continuous variables, and Fisher's exact test for binary variables. cMedian (range). dDue to the paired design of the trial, by definition 50% of all kidneys were machine perfused, and 50% were cold stored. Hence, no statistical tests were performed for these baseline characteristics.**

## SERUM ANGIOPOIETIN AND POSTTRANSPLANT OUTCOME

Figure 3 shows Kaplan-Meier curves for one year death-censored graft survival stratified into ang-2 under vs. above the median (4.7 ng/ml). One year graft survival in the group with high ang-2 was 95% vs. 89% in the low ang-2 group (p=0.003). In

a multivariate Cox regression analysis (table 2), ang-2 was identified as an independent prognostic factor for one year death censored graft survival, reducing the risk of graft failure with a hazard ratio of 0.91 (p=0.027). In contrast, ang-1 did not predict graft survival. Also, in a univariate analysis both ang-1 and ang-2 showed no predictive value for DGF and PNF. Therefore, no multivariate models were constructed for these associations.

| **Variable**<br>**Graft loss** | **Hazard ratio**<br>**(95% CI)** | **P-value** |
|---|---|---|
| Machine perfusion vs. cold storage | 0.628 (0.347-1.134) | 0.12* |
| HLA mismatches — no. | 1.221 (0.970-1.536) | 0.089 |
| DCD donor vs. DBD donor | 0.918 (0.328-2.574) | 0.870 |
| Donor age — yr | 1.053 (1.020-1.088) | 0.0014 |
| Recipient age — yr | 0.972 (0.949-0.996) | 0.024 |
| Panel-reactive antibody level — % | 1.012 (0.995-1.029) | 0.180 |
| Second or later transplantation vs. first transplantation | 1.160 (0.677- 1.986) | 0.590 |
| Cold ischemic time — hr | 1.011 (0.953-1.072) | 0.720 |
| Duration of pretransplantation dialysis — yr | 1.003 (0.869-1.157) | 0.970 |
| Trauma vs. other cause of death | 1.268 (0.393-4.086) | 0.690 |
| Cranial bleeding vs. other cause of death | 1.639 (0.621-4.325) | 0.320 |
| Brain ischemia vs. other cause of death | 1.149 (0.295-4.475) | 0.840 |
| Duration of stay on intensive care unit before death — days | 1.076 (1.015-1.142) | 0.014 |
| Angiopoietin 2 — ng/ml | 0.908 (0.834-0.989) | 0.027 |

**Table 2 Multivariate analysis of the risk of graft loss.**

**A Cox proportional hazards model was used to determine the hazard ratio for graft failure. Hazard ratios are associated with a 1-unit increase in each covariate. CI denotes confidence interval, DBD donation after brain death, DCD donation after cardiac death. Data on graft survival were censored at the time of death in patients who died with a functioning allograft. * In the original clinical trial, MP vs. CS was associated with a significant reduction in the risk of graft failure in a similar Cox model. It is most likely that the present analysis did not pick up this significant association due to fewer available cases that could be included into the model (594 instead of 672 recipients).**

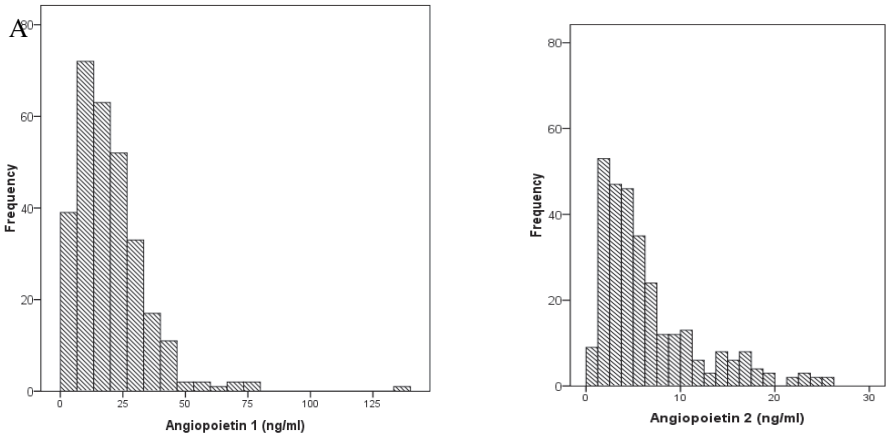
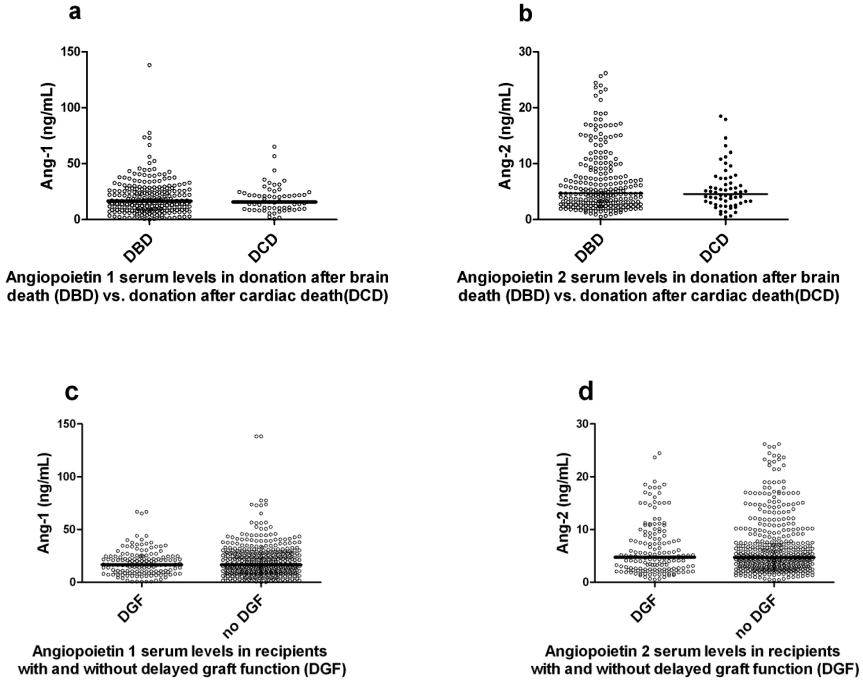



**Figure 1 Histogram of distribution of serum levels of angiopoietin 1 a and angiopoietin 2 b**



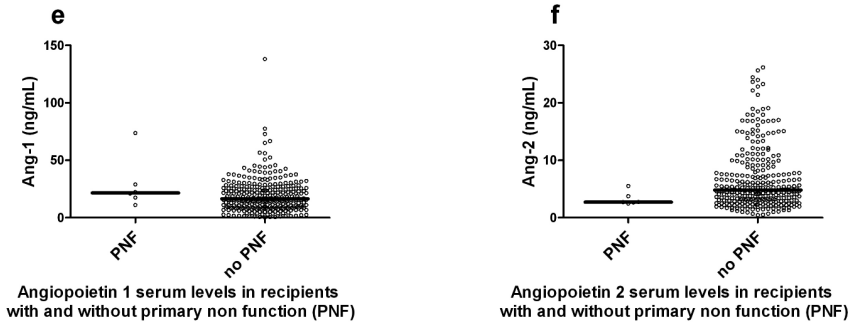



**Figure 2 Dot plots of serum angiopoietin 1 and angiopoietin 2 levels in donation after brain death vs. donation after cardiac death a-b; in recipient with and without delayed graft function c-d and recipients with and without primary non function e-f.**

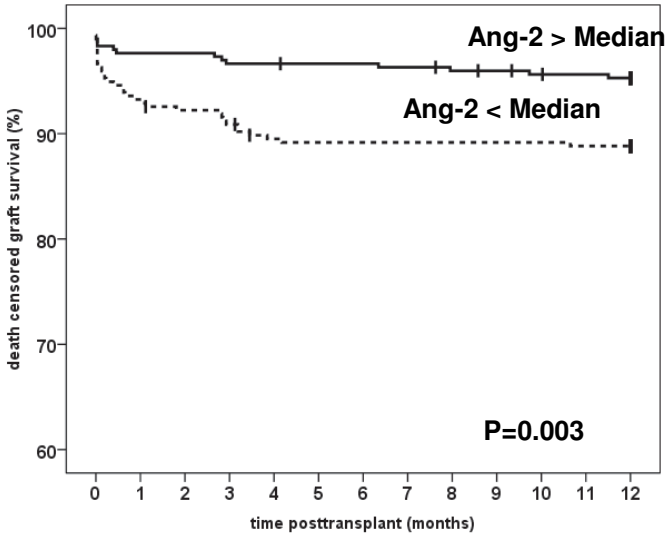



**Figure 3 Kaplan-Meier curves of death censored graft survival stratified into high and low angiopoietin 2 concentration in the donor. Under versus above the median; Log rank test p=0.003**

## Discussion

This study shows that circulating serum angiopoietin 2 in the donor has an independent predictive value for transplant outcome in the recipient Results were obtained in the context of a large randomized trial in kidney donation and transplantation (18).

Transplant clinicians are often confronted with the dilemma whether or not to accept an organ offer for a certain patient on the waiting list. So far, no donor serum biomarker proved to be a true independent predictor of posttransplant

outcome in the context of other strong predictors such as donor type, donor age, number of previous transplants, HLA mismatch, and cold ischemic time. The availability of a potent biomarker will help the clinician to maximize the total donor pool.

At present, we feel that there is no rationale for setting a hard cut-off value of any biomarker to determine acceptance or discard of a kidney. However, among the other prognostic factors, ang-2 can add relevant information to the clinical decision-making algorithm by which a physician determines whether a kidney will be suitable for transplant. Future research should yield better insight into relevant cut off points to aid decision making, taking into account important factors such as donor and recipient age, duration of dialysis, or the length of stay at the intensive care unit. So far, no clinically validated ang-2 test has been available. However, with the use of ELISA or other laboratory techniques, we expect that a rapid and clinically validated assay can be designed to be used in transplantation practice.

The physiological and molecular basis for this association between ang-2 in the donor and graft survival in the recipient remains to be unravelled. Angiopoietins play an early role in the first hour of the inflammation process, whereas their long term effects are associated with vascular remodelling (12). In inflammation, ang-1 acts as a stabilizer of the vasculature and ang-2 promotes vascular leakage (13). In several clinical settings such as profound trauma, acute respiratory distress syndrome, and kidney disease, ang-2 has been independently associated with patient outcome (14-16). In pregnancy, an increase of circulating ang-2 is needed to prevent preeclampsia (12). Although the exact mechanism has not yet been completely elucidated, the angiopoietin system seems to have a distinct regulatory function in acute inflammation and structural remodelling. Basic research, e.g. with cell culture studies, is needed to provide better understanding of our results. Several important proteins act in concert with the angiopoietin Tie system. Tumor necrosis factor, interleukin-1, vascular endothelial growth factor (VEGF), and fibroblast growth factor, in combination with microenvironmental factors (such as hypoxia) all have a role in this cascade (18).

In conclusion, this study showed that a decreased donor serum angiopoietin-2 is independently associated with graft loss in the recipient. Therefore, it can be used as an extra clinical tool to assess renal graft quality before transplantation. In the future, therapeutic interventions that modulate the angiopoietin response in the donor could be a novel tool to improve organ quality. The exact focus of such interventions, as well as the molecular mechanisms that govern angiopoietin responses in a deceased donor offer an interesting new topic for further research.

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