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## Complement modulation in renal replacement therapy

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# Chapter 8

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## **C1-inhibitor treatment decreases renal injury in an established brain-dead rat model.**

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## **Abstract**

### **Background**

Kidneys derived from brain-dead (BD) donors have lower graft survival rates compared to kidneys from living donors. Complement activation plays an important role in brain death. The aim of our study was therefore to investigate the effect of C1-inhibitor (C1-INH) on BD-induced renal injury.

### **Methods**

Brain death was induced in rats by inflating a subdurally placed balloon catheter. Thirty minutes *after* BD, rats were treated with saline, low-dose or high-dose C1-INH. Sham-operated rats served as controls. After 4 hours of brain death, renal function, injury, inflammation and complement activation was assessed.

### **Results**

High-dose C1-INH treatment of BD donors resulted in significantly lower renal gene expression and serum levels of IL-6. Treatment with C1-INH also improved renal function and reduced renal injury, reflected by the significantly lower KIM-1 gene expression and lower serum levels of LDH and creatinine. Furthermore, C1-INH effectively reduced complement activation by brain death and significantly increased functional levels. However, C1-INH treatment did not prevent renal cellular influx.

### **Conclusions**

Targeting complement activation after the induction of brain death reduced renal inflammation and improved renal function, before transplantation. Therefore, strategies targeting complement activation in human BD donors might clinically improve donor organ viability and renal allograft survival.

## Introduction

Today, brain-dead (BD) donors still comprise the majority of deceased organ donors. It is well known that fully mismatched living donors have better early graft function and improved long-term outcome compared to kidneys from fully matched deceased donors.<sup>1</sup> This illustrates that donor origin is an important cause of allograft injury. Donor brain death is characterized by hemodynamic and hormonal instabilities resulting in immune activation and eventually decreased organ viability before and after transplantation.<sup>2,3</sup> Therefore, attenuating BD-induced renal injury in the donor seems to be a promising therapeutic strategy to improve outcome after renal transplantation.<sup>3,4</sup>

The harmful effect of donor brain death on renal allograft survival is in part explained by immunological changes.<sup>5,6</sup> BD donors have increased levels of cytokines (IL-1 $\beta$ , IL-6, IL-8), chemokine's (MCP-1) and adhesion molecules (ICAM-1, VCAM-1, E- and P-selectin).<sup>7</sup> Subsequently, increased numbers of infiltrating macrophages (M $\Phi$ ) and polymorphonuclear (PMN) cells are found in renal tissue of BD donors.<sup>8,9</sup> Ultimately, this leads to tissue damage and repair as reflected by increased expression of KIM-1, a marker of BD-induced renal injury.<sup>10</sup> Consequently, recipients of renal allografts from BD donors have a higher chance to develop delayed graft function (DGF, defined as the need for dialysis within the first 7 days post-transplant) and a lower long-term renal graft survival.<sup>1,11</sup>

There is circumstantial evidence for a prominent role of complement activation in the pathogenesis of renal injury in deceased donors.<sup>12</sup> In previous studies, we demonstrated that circulating complement components are activated in deceased donors and are associated with acute rejection after renal transplantation.<sup>13</sup> Furthermore, in kidneys from BD donors, complement component 3 (C3) was upregulated and activated before kidney retrieval without additional expression after cold ischemia or reperfusion.<sup>14</sup> Moreover, microarray analysis of 554 kidney biopsies showed enrichment of complement pathways in BD donors prior to organ retrieval.<sup>15</sup> In addition, genetic variation of donor C3 was associated with allograft outcome of deceased donors.<sup>16,17</sup> Altogether, these data demonstrate that complement activation plays a pivotal role in BD-associated renal injury and may impact long term allograft survival rates. In accordance with this, inhibition of the complement system in rat BD donors was shown to improve renal allograft function in the recipient after transplantation.<sup>18</sup>

C1 esterase inhibitor (C1-INH) is one of the central regulators of the complement system, as it interacts with different complement factors and additionally plays a key role in the kinin- and coagulation system.<sup>19</sup> Previously, it has been shown that C1-INH is able to inhibit complement activation *in vitro* of the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP).<sup>20-22</sup> The clinical value of C1-INH has been well established since it has been used extensively for the prophylaxis and treatment of hereditary angioedema (HAE).<sup>23</sup> Preliminary clinical studies in transplantation show the therapeutic potential of C1-INH in the treatment of renal ischemia-reperfusion injury and antibody-mediated rejection.<sup>24</sup>

The aim of our study was to investigate the effect of C1-INH on BD-induced renal injury. Rats were treated after the induction of brain death since it is analogous to a potential clinical intervention strategy that would involve treating human BD donors prior to procurement for transplantation.

## Materials and Methods

### *Animals*

Adult male Fisher F344 rats (260–300 g) were used. Animals received care in compliance with the guidelines of the local animal ethics committee according to Experiments on Animals Act (1996) issued by the Dutch Ministry of Public Health, Welfare and Sports.

### *Experimental protocol*

Nanofiltered plasma derived C1-inhibitor (Cinryze®) was provided by ViroPharma, Exton, USA. Brain death was induced as described previously.<sup>25</sup> In short: animals were anesthetized, intubated and ventilated. A baseline blood sample (100 µl) was drawn. Temperature and blood pressure were continuously monitored and regulated. Through a frontolateral borehole in the skull, a catheter was placed subdural and inflated slowly. The induction was completed after 30 minutes and the rats were brain dead. After another 30 minutes, a blood sample (100 µl) was taken (before treatment). During brain death, animals received no anesthesia. If blood pressure fell below 80 mm Hg, it was restored by the administration of 500 µl of 10% hydroxyethyl starch solution (HAES) or 0.01 mg/ml of noradrenaline (Supplementary data). After four hours of brain death, blood was collected from the aorta and organs were flushed with cold saline and retrieved. Retrieved organs were paraffin embedded or snap frozen in liquid nitrogen stored at –80°C together with collected serum. In the sham-operated group, a hole was drilled in the skull without insertion of the balloon catheter and animals were ventilated for half an hour under anesthesia before animals were sacrificed (Figure 1). Rats were randomly divided into six groups:

**Group 1:** sham-operated rats (n=4) treated with saline.

**Group 2:** sham-operated rats (n=4) treated with a 100 U/kg of C1-INH (=low-dose).

**Group 3:** sham-operated rats (n=4) treated with a 400 U/kg of C1-INH (=high-dose).

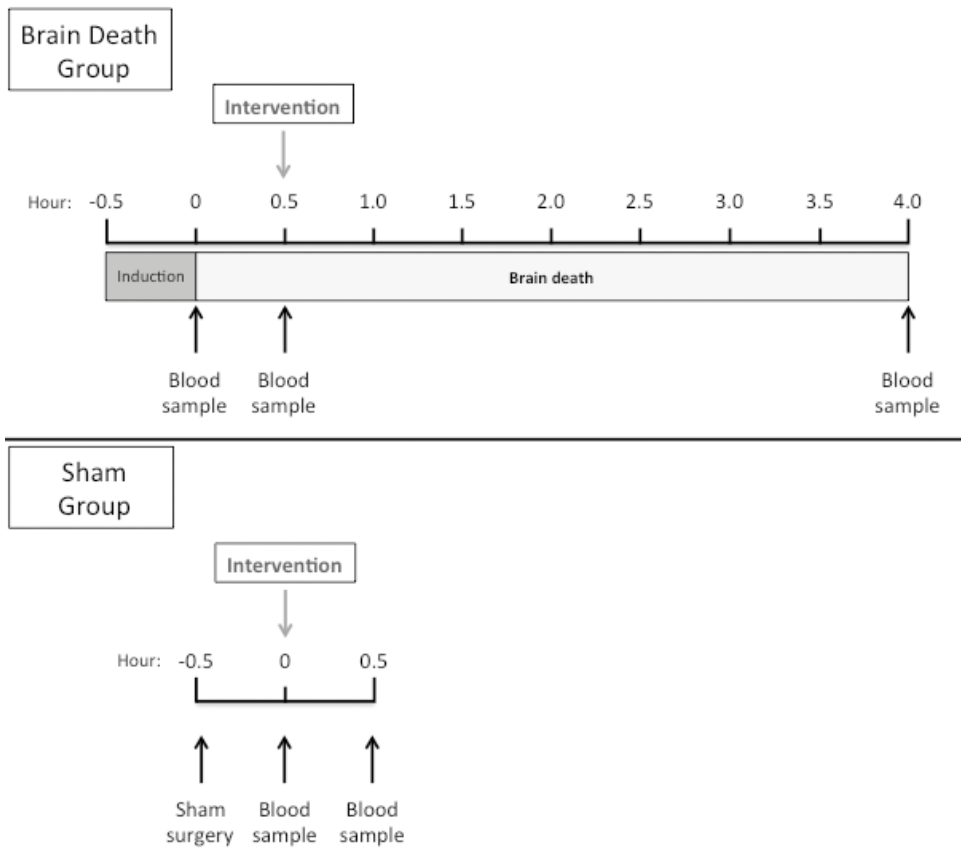
**Group 4:** BD rats (n=10) treated with saline.

**Group 5:** BD rats (n=10) treated with a 100 U/kg of C1-INH (=low-dose), 0.5 hour after BD.

**Group 6:** BD rats (n=10) treated with a 400 U/kg of C1-INH (=high-dose), 0.5 hour after BD.

All treatments were administered via the femoral vein and the volume of the treatment (500 µl) was equal between the different groups. After the confirmation of BD, the mean arterial pressure was kept above 80 mmHg in all animals throughout the experiment (supplementary data).

**Figure 1**  
Schematic presentation of the protocol in brain-dead rats and sham-operated rats.



*In the brain-dead (BD) group, a catheter was placed subdural through a frontolateral borehole in the skull and inflated slowly. After 30 minutes rats were brain death another 30 minutes later BD rats were treated with saline, a low-dose of C1-INH (100 U/kg) or a high-dose of C1-INH (400 U/kg). After four hours of brain death, animals were sacrificed and organs were collected. Blood samples were collected at baseline, 30 min after BD (before intervention) and after 4 hours of BD. In the sham group, a hole was drilled in the skull without insertion of the balloon catheter and animals were ventilated for half an hour under anesthesia before animals were sacrificed. Directly after sham surgery rats were treated with saline, low-dose or high-dose of C1-INH. Blood samples were collected at baseline (before intervention) and 30 min after sham surgery.*

### **RNA isolation and qPCR**

Total RNA was extracted from rat kidneys using the Trizol reagent method (Invitrogen, Breda, the Netherlands). The isolated RNA was then treated with DNase I to remove any contamination with genomic DNA (Invitrogen, Breda, the Netherlands). The integrity of isolated RNA was analyzed by using gel electrophoresis. Complementary DNA (cDNA) was synthesized from 1- $\mu$ g total RNA using the M-MLV (Moloney murine leukemia virus) Reverse Transcriptase enzyme kit (Invitrogen), oligo dT primers and RNase inhibitor (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. Primer sequences (Table 1) were designed based on Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Amplification and detection were performed with the ABI Prism 7900-HT Sequence Detection System (Applied Biosystems) using emission from SYBR green master mix

(Applied Biosystems). All samples for RT-PCR were done in triplicate. The expression of genes was normalized to  $\beta$ -actin. The data are presented as fold increase compared to the housekeeping gene. Fold increase of the sham-operated rats treated with saline was set at 1; the other groups were calculated accordingly.

**Table 1** Gene-specific qPCR primers and their respective PCR fragment lengths.

Primers:	Primer sequences	Amplicon length (bp):
$\beta$ -actin	5'-GGAAATCGTGCGTGACATTA-3' 5'-GCGGCAGTGGCCATCTC-3'	74
BAX	5'-GCGTGGTTGCCCTTCTAC-3' 5'-TGATCAGCTCGGGCACTT-3'	74
BCL2	5'-CTGGGATGCCTTGTGGAA-3' 5'-TCAGAGACAGCCAGGAGAAATCA-3'	70
IL-6	5'-CCAACCTCCAATGCTCTCTAATG-3' 5'-TTCAAGTGTTC-3'	89
P-selectin	5'-TCTCTGGGTCTTCGTGTTCTTATCT-3', 5'-GTGTCCCTAGTACCATCTGAA-3'	80
VCAM-1	5'-TGTGGAAGTGTGCCGAAA-3' 5'-ACGAGCCATTAACAGACTTAGCA-3'	84
MCP-1	5'-CTTTGAATGTGAACTTGACCCATAA-3' 5'-ACAGAAGTGCTTGAGGTGGTTGT-3'	78
KIM-1	5'-AGAGAGAGCAGGACACAGGCTT-3' 5'-ACCCGTGGTAGTCCCAAACA-3'	75

### Immunohistochemistry

Frozen sections were fixed with acetone, followed by endogenous peroxidase blocking. Paraffin embedded tissue sections were deparaffinized and antigen retrieval was performed, followed by endogenous peroxidase blocking. Sections were incubated with a primary antibody for 1h at RT (Table 2). After washing, sections were incubated with appropriate secondary and tertiary antibodies (Dako, Glostrup, Denmark). The reaction was developed by the addition of 3-amino-9-ethylcarbazole (AEC) and counterstained with hematoxylin solution (Merck, Darmstadt, Germany).

For HIS48 and ED-1 (for granulocytes and macrophages respectively), in each section the number of glomerular and interstitial positive cells was assessed as the average count from 20 different microscopic fields, randomly divided throughout the renal cortex, using a light microscope (objective 200x).

**Table 2** Primary antibodies used for Immunohistochemistry.

Specificity	Sections	Antibody	Supplier	Sec/Tert antibodies
HIS 48	Frozen	Mouse monoclonal anti-rat granulocytes	IQProducts, Groningen, The Netherlands	R $\beta$ $\alpha$ M <sup>PO</sup> /G $\alpha$ Rb <sup>PO</sup>
ED-1	Paraffin	Mouse monoclonal anti-rat macrophages/ monocytes (ab31630)	Abcam, Oxford, UK	R $\beta$ $\alpha$ M <sup>PO</sup> /G $\alpha$ Rb <sup>PO</sup>

### ***Biochemical determinations***

Creatinine and lactate dehydrogenase (LDH) were determined in serum samples in a routine fashion (Mega, Merck, Amsterdam, The Netherlands).

### ***Complement activation and functional levels***

Rat complement pathway activity was assessed by functional complement ELISAs for the CP, LP, and AP. The sC5b-9 ELISA was used for the detection of complement activation.<sup>26</sup> Per rat the baseline values were set at a 100% or 1 AU/mL and consecutive samples were calculated accordingly.

### ***IL-6 ELISA***

IL-6 production in serum was determined using Rat IL-6 Quantikine ELISA Kit (R&D Systems, Abingdon, UK) according to the supplied protocol.

### ***Statistics***

Statistical analysis was performed with StatsDirect (v 3.0.133, Cheshire, UK). The Kruskal–Wallis test and Mann–Whitney U test were used to assess differences between groups of non-parametric data and one-way analysis of variance and t test for normally distributed data. The Dwass-Steel-Critchlow-Fligner test was then used to determine where the differences lay. All statistical tests were 2-tailed with  $P < 0.05$  regarded as significant. Results are presented as a median and interquartile range for non-parametric data and mean and SEM for parametric data.

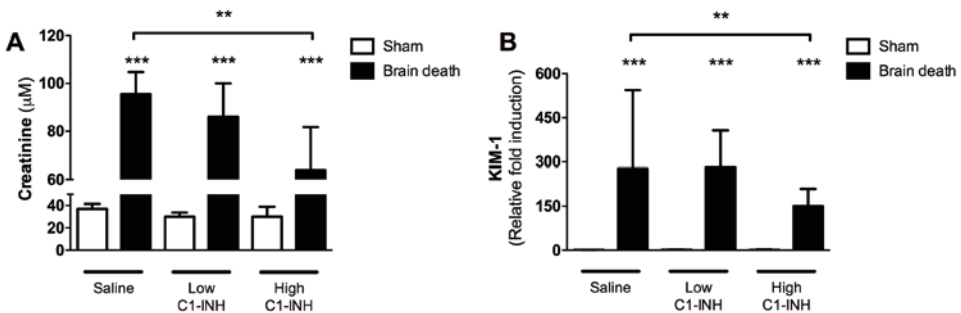


## Results

### *Treatment with C1-INH protects kidneys from brain death-induced injury*

In BD rats creatinine levels were significantly higher after 4h compared to sham-operated animals, indicating reduced renal function (Figure 2A,  $P < 0.001$ ). In accordance with these observations, renal mRNA expression of KIM-1 was also significantly upregulated in kidneys from BD rats compared to controls (Figure 2B,  $P < 0.001$ ). Since complement is known to play a role in BD-induced renal injury, we investigated the effect of C1-INH treatment on kidneys of BD donors. High-dose C1-INH treatment significantly reduced creatinine levels (Figure 2A). Median creatinine levels in BD rats treated with high-dose C1-INH were  $64 \mu\text{M}$ , compared to  $96 \mu\text{M}$  in saline treated BD rats ( $P < 0.01$ ). Sham-operated rats had a median creatinine of  $34 \mu\text{M}$ . In accordance, high-dose C1-INH treatment reduced renal injury in BD rats as seen by the lower mRNA expression of KIM-1 (Figure 2B). KIM-1 expression in BD kidneys was 47% lower in the C1-INH treated rats compared to untreated rats ( $P < 0.01$ ). Low-dose C1-INH treatment was unable to improve renal function or injury.

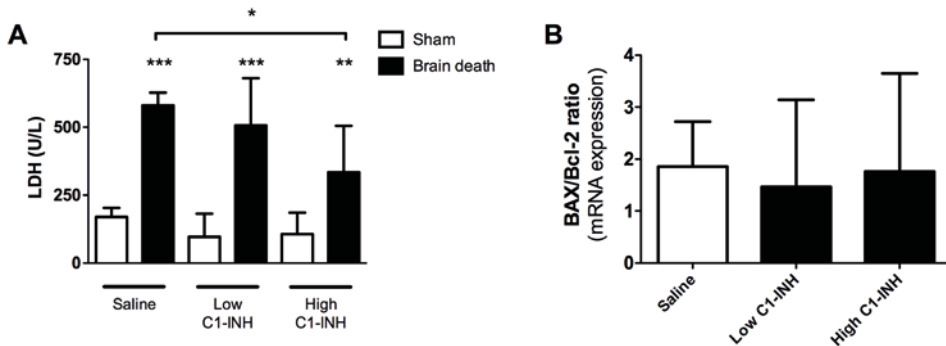
**Figure 2**  
C1-inhibitor treatment in brain-dead rats reduced renal injury.



(A) Plasma creatinine levels of brain-dead (BD) or sham-operated rats treated with saline, 100 U/kg (low) C1-INH or 400 U/kg (high) C1-INH. (B) KIM-1 gene expression in kidneys of rats after BD or sham operation. Quantitative real-time RT-PCR was performed. The mRNA expression of KIM-1 relative to  $\beta$ -actin was set at 1 in sham-operated rats treated with saline and the other values are calculated accordingly. Data are shown as median and interquartile range and were analyzed by Kruskal Wallis test with an option for multiple comparisons (\*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Asterisks above the bars denote significant differences between BD and sham animals of the same treatment, while asterisks above the capped line indicate significant differences between BD groups from the different treatments. The number of rats used is 10 per BD group and 4 per sham-operated group.

Furthermore, BD rats had significantly higher levels of LDH compared to the sham group ( $P < 0.001$ ). High-dose C1-INH treatment led to significant lower LDH levels compared to the untreated BD rats (Figure 3A,  $P < 0.05$ ). On the other hand, BD-induced renal apoptosis was not altered by C1-INH treatment since the Bcl2/BAX-ratio was not significantly different between the groups (Figure 3B).

**Figure 3**  
C1-inhibitor treatment reduces tissue damage in brain-dead rats but not renal apoptosis.



(A) Plasma LDH levels of brain-dead (BD) or sham-operated rats treated with saline, 100 U/kg (low) C1-INH or 400 U/kg (high) C1-INH. (B) BAX/Bcl-2 gene expression ratio in kidneys of rats after brain death. Quantitative real-time RT-PCR was performed. The ratio was calculated by dividing BAX mRNA expression by Bcl-2 mRNA expression. Data are shown as median and interquartile range and were analyzed by Kruskal Wallis test with an option for multiple comparisons (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Asterisks above the bars denote significant differences between BD and sham animals of the same treatment, while asterisks above the capped line indicate significant differences between BD groups from the different treatments.  $N$  is 10 for BD groups and  $N$  is 4 for sham groups.

#### **Brain death leads to local and systemic complement activation, resulting in complement consumption.**

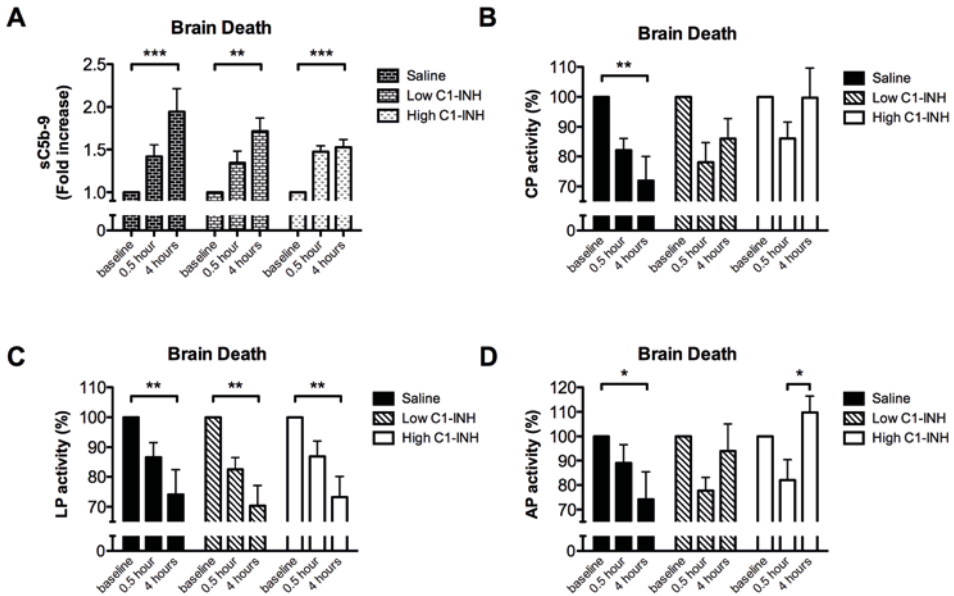
Next, we investigated complement activation and functional levels during brain death. The sC5b-9 levels increased significantly during brain death from 1.00 AU/mL at baseline to 1.95 AU/mL after 4 hours (Figure 4A,  $P < 0.001$ ). Between 0.5 and 4 hours after BD induction the sC5b-9 levels rose as well although not statically significant (0.5h: 1.42 AU/mL, 4h: 1.95 AU/mL). In sham-operated animals, there was no significant increase in sC5b-9 levels (supplementary data). Subsequently, we assessed whether treatment with C1-INH was able to prevent systemic complement activation in rats when administered 0.5 hour after BD. High-dose C1-INH treatment prevented further increase between 0.5 and 4 hours in BD rats (0.5h: 1.47 AU/mL, 4h: 1.53 AU/mL). However, after 4 hours sC5b-9 levels were still significantly higher than baseline samples in the C1-INH treated BD rats.

We next determined whether the systemic complement activation was accompanied by complement depletion by measuring functional complement levels. In BD rats functional levels were significantly lower after 4 hours compared to baseline levels for all three pathways ( $P < 0.05$ ). After 4 hours, functional levels were 72%, 74% 74% for the CP, LP, and AP, respectively (Figure 4B-D). There was a drop in functional levels for all pathways between 0.5 and 4 hours as well, however, this was not significant. In the control group, functional levels of the LP and AP were not significantly altered by the sham-operation (supplementary data). Nonetheless, there was a significant decrease in the functional levels of the CP in the sham-operated animals (15% reduction compared to baseline,  $P < 0.05$ ). Next, we determined whether treatment with C1-INH prevented complement consumption in rats when administered 0.5 hour after BD induction. For the CP, functional levels rose between 0.5 and 4 hours after both low-dose and high-dose C1-INH treatment (Figure 4B). After 4 hours functional levels were no longer significantly lower compared to baseline. In the sham-operated rats, high-dose

C1-INH significantly increased CP functional complement levels as well (supplementary data). For the AP a similar protection was seen after both low-dose and high-dose C1-INH treatment (Figure 4D). Strikingly, high-dose C1-INH treatment even significantly increased functional AP levels compared to baseline ( $P < 0.05$ ), indicating prevention of complement consumption of the AP. For the LP, the significant decrease in functional levels by BD could not be prevented by C1-INH (Figure 4C).

**Figure 4**

C1-inhibitor treatment prevented further complement activation and consumption of the classical and alternative pathway, but not the lectin pathway.



Plasma soluble C5b-9 (sC5b-9) levels (A) and functional complement levels for the classical pathway (B), lectin pathway (C) and alternative pathway (D) of brain-dead (BD) rats. Levels were determined at baseline ( $t=0$  hour), prior to treatment ( $t=0.5$  hour) and prior to sacrifice ( $t=4$  hours). Values at baseline are set at 1.0 or 100% per rat, and other consecutive levels are calculated accordingly. Data are expressed as mean percentage  $\pm$  SEM and analyzed by ANOVA with a Bonferroni posthoc test. Significant differences are indicated (\* $P < 0.05$ ; \*\* $P < 0.01$  and \*\*\* $P < 0.001$ ).  $N$  is 10 for BD groups; except for the saline treated BD rats ( $N = 9$ ).

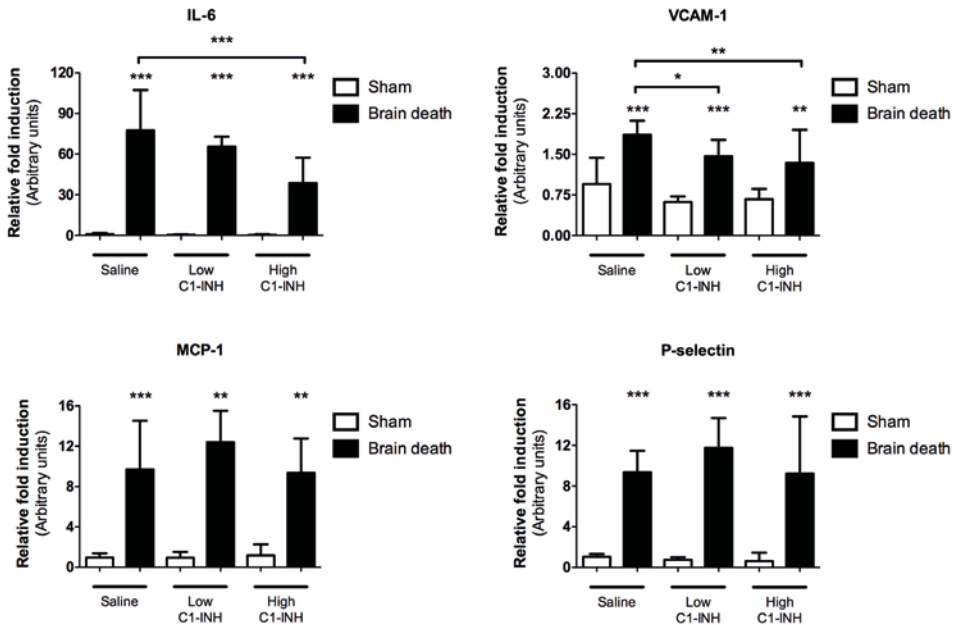
### **C1-INH treatment reduces both renal IL-6 expression and systemic IL-6 levels.**

Subsequently, we assessed the gene expression of pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ), chemokine's (MCP-1), and adhesion molecules (VCAM-1, ICAM-1, E-selectin and P-selectin). In BD rats, renal expression levels of IL-6, MCP-1, VCAM-1, and P-selectin were significantly increased compared to sham operated rats (Figure 5). No significant increase was found for renal expression of IL-1 $\beta$ , TNF-alpha, E-selectin and ICAM-1 in BD rats (data not shown). Next, we evaluated the effect of C1-INH treatment on the expression of IL-6, MCP-1, VCAM-1, and P-selectin. Treatment with both high-dose and low-dose C1-INH significantly reduced VCAM-1 expression (Figure 5A). A clear downward trend was visible for IL-6 expression in the BD rats treated with C1-INH compared to untreated animals (Figure 5B). In BD rats with high-dose C1-INH renal expression of IL-6 was reduced

by 55% ( $P<0.001$ ). Serum levels of IL-6 were also determined to assess whether systemic levels of IL-6 were also lower in C1-INH treated animals (Figure 6). Levels of IL-6 were not determined in the sham groups. The median IL-6 serum concentration was 107 pg/mL in high-dose C1-INH treated rats compared to 206 pg/mL in BD controls ( $P<0.05$ ).

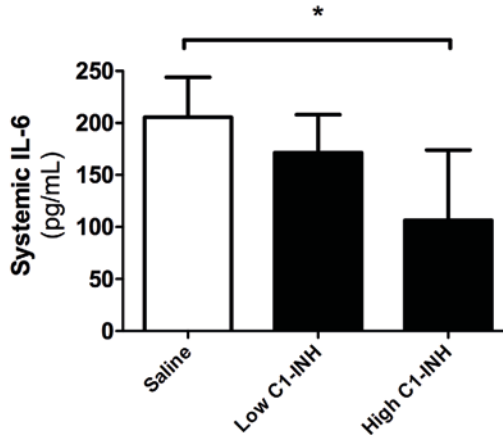
To further characterize local renal inflammation upon brain death, infiltrating neutrophils (PMN) and macrophages (M $\Phi$ ) were quantified by immunohistochemistry. There was a significant increase in the number of infiltrating PMN but not in M $\Phi$  in the renal cortex of BD rats compared to the sham-operated group ( $P<0.01$ ). Treatment with C1-INH, low or high-dose, did not attenuate the increase in infiltrating PMN in the renal cortex (Figure 7A). However, treatment with C1-INH in the BD rats appears to lead to increased number of M $\Phi$  compared to BD controls and C1-INH sham-operated rats (Figure 7B).

**Figure 5**  
Reduction in renal gene expression of inflammatory mediators in brain-dead rats after C1-inhibitor treatment.



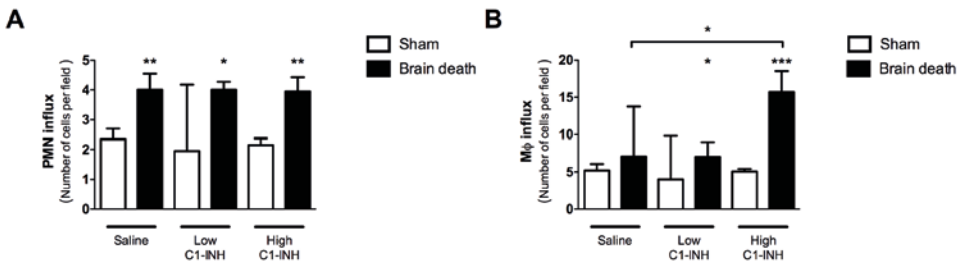
Gene expression of inflammatory markers in kidneys of brain-dead (BD) or sham operated rats, determined by quantitative real-time RT-PCR. mRNA expression of (A) IL-6, (B) VCAM-1, (C) MCP-1, (D) P-selectin. Data are shown as expression relative to  $\beta$ -actin, were sham-operated rats treated with saline are set at 1 and the rest is calculated accordingly. Data are shown as median and interquartile range and were analyzed by Kruskal Wallis test with an option for multiple comparisons ( $*P<0.05$ ,  $**P<0.01$ ,  $***P<0.001$ ). Asterisks above the bars denote significant differences between BD and sham animals of the same treatment, while asterisks above the capped line indicate significant differences between BD groups from the different treatments.  $N$  is 10 for BD groups and  $N$  is 4 for sham groups.

**Figure 6**  
C1-inhibitor treatment led to reduced systemic IL-6 in brain-dead rats.



Serum levels of Interleukin-6 (IL-6) in brain-dead (BD) rats, treated with saline or C1-INH. Data are shown as median and interquartile range and were analyzed by Mann-Whitney U test (\* $P < 0.05$ ).  $N$  is 10 per groups.

**Figure 7**  
C1-inhibitor did not decrease the number of infiltrating granulocytes or macrophages in the renal cortex of brain-dead rats.



Renal influx of polymorphonuclear leukocytes (PMN) and macrophages (Mφ) was evaluated by immunohistochemistry. The HIS48 monoclonal antibody reacts with a molecule expressed on the surface of all rat granulocytes. The monoclonal antibody ED1 is a marker for rat macrophages. In each section the number of interstitial positive cells was assessed by the average count from 10 different microscopic fields, randomly divided throughout the renal cortex, using a light microscope (objective 200x). Data are shown as median and interquartile range and were analyzed by Kruskal Wallis test with an option for multiple comparisons (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ). Asterisks above the bars denote significant differences between BD and sham animals of the same treatment, while asterisks above the capped line indicate significant differences between BD groups from the different treatments.  $N$  is 10 for BD groups and  $N$  is 4 for sham groups.

## Discussion

In this study, we demonstrated that complement inhibition in BD donors attenuates renal injury before organ retrieval. In our model, animals were treated after the induction of brain death, since this closely resembles the potential clinical situation that would involve treating donors after the diagnosis of brain death. We found that high-dose C1-INH treatment after brain death induction decreased local renal and

systemic inflammation and renal injury and improved renal function in the donor. Therefore, strategies targeting complement activation in human BD donors might improve donor organ viability and renal allograft survival. The ideal timing to target complement activation would be in the intensive care unit, shortly after confirmation of brain death. Potentially, complement inhibition in the donor could be more effective than in the recipient prior to reperfusion injury, since complement is already activated in the donor. In humans, randomized clinical trials have to explore whether complement inhibition in brain-dead donors alone or in combination with treatment of the recipient would improve renal allograft outcome after kidney transplantation.

Donor organ injury induced by brain death is considered to be an important risk factor for DGF, renal allograft graft rejection and allograft survival after transplantation.<sup>1,11,27–29</sup> The complement system has been shown to play an important role in BD-induced renal injury.<sup>13,14,30</sup> In addition, treatment with complement inhibiting drugs has proven to be beneficial in preclinical models of DGF,<sup>18</sup> emphasizing that strategies targeting complement activation are potentially beneficial in the transplantation setting. C1-INH is a complement inhibitor of all three pathways,<sup>20–22</sup> and this already-available therapeutic agent is currently used for the treatment of HAE. C1-INH treatment has already been shown to improve outcome in a number of disease models, including ischemia-reperfusion injury, antibody-mediated transplant rejection, and xenotransplantation models.<sup>43</sup> The use of C1-INH may have greater benefits for BD donors than other complement inhibitors. Plasma-derived C1-INH appears to be extremely safe since it has been used for over three decades.<sup>31</sup> C1-INH also regulates the coagulation system and can inhibit leukocyte adhesion to the endothelium, which also contributes to BD-induced renal injury.<sup>15,32,33</sup> It has been suggested that C1-INH has additional anti-inflammatory functions and anti-apoptotic effects independent of protease inhibition.<sup>40–42</sup> C1-INH also regulates the coagulation and the kallikrein-kinin systems, which also contributes to BD-induced organ injury.<sup>15,32,34</sup> A vital consideration for any intervention in BD donors is that it should not impact the successful utilization of other organs. This is a potential limitation of complement inhibition in the donor since it is not known if all donor organs would benefit from such therapy. Nevertheless, C1-INH treatment has already shown promising results in lung transplantation.<sup>35</sup> In addition to the trials for the treatment of renal ischemia-reperfusion injury and renal antibody-mediated rejection,<sup>36–40</sup> C1-INH treatment is also being tested in liver transplantation (NCT 01886443 and 02251041).<sup>24</sup> In accordance, in our study C1-INH did not negatively impact the liver since specific injury markers, AST and ALT were unaltered (supplementary data). Lastly, C1-INH inhibits at an early point and could thereby decrease organ injury, whereas later blockage can only eliminate effector functions of complement activation.

The cause of the immune activation in BD donors is not well understood but it is thought to be initiated by damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) in the systemic circulation as well as in the local environment (41). Studies on the contribution of complement in brain death have mostly focused on local C3 expression and synthesis.<sup>32,42,43</sup> However, we show that brain death induces systemic complement activation already after 0.5 hour. Between 0.5 and 4 hours of BD, there was a further stepwise increase of sC5b-9. Furthermore, induction of brain death led to significant lower functional levels of all three pathways, which reflects significant systemic complement activation. This is in line with findings in human BD

donors that showed increased plasma levels of sC5b-9, C4d and Bb.<sup>13</sup> In our animal model, significant complement consumption of the CP and AP in BD rats was prevented by treatment with high-dose C1-INH, thereby increasing functional levels of these pathways. No effect of C1-INH treatment could be seen on the LP. However, human C1-INH was used to inhibit complement in rats and the biggest discrepancies among the complement pathways between rodents and humans are found in the LP.<sup>44</sup> Altogether, these data show that high-dose C1-INH prevented the further increase of sC5b-9 by BD and thereby increased functional levels of the CP and AP. This excludes the possibility that the *in-vivo* administered C1-INH is interfering with the *in-vitro* activity assay. Furthermore, the concentration of C1-INH used here is comparable to previous studies in pigs and human.<sup>36,40</sup>

Our study shows that renal inflammation is significantly reduced after treatment of BD rats with C1-INH. High-dose C1-INH treatment partly prevented the BD-induced gene expression of IL-6 and VCAM-1. Furthermore, also systemic IL-6 was significantly reduced by C1-INH treatment. These findings suggest that complement inhibition by C1-INH protects allografts from the development of inflammation. However, we did not assess systemic levels of TNF- $\alpha$  and IL-1 $\beta$ , since previous studies of this model have shown that IL-6 is the cytokine that is significantly elevated, opposed to other prominent pro-inflammatory markers such as IL-1 $\beta$  and TNF- $\alpha$ .<sup>8</sup> Additionally, IL-6 has been shown to be an important mediator in the pro-inflammatory reaction of BD.<sup>45-48</sup> Recently it was demonstrated that higher levels of IL-6 in the donor are associated with a worse outcome in the recipient.<sup>49</sup> Thus, C1-INH treatment leads to a better renal function in BD donors, which could be ascribed to a consequence of improved organ allograft quality by reducing inflammatory cytokines IL-6. C1-INH significantly reduced creatinine levels in our BD model before organ retrieval. Yet, creatinine levels remained significantly higher than sham-operated animals. Hemodynamic and hormonal instabilities should also be considered in brain death since these changes additionally affect renal function. KIM-1 expression was also significantly lower by C1-INH treatment. KIM-1 has been shown to be a specific marker of BD-induced kidney damage. In human BD donors, urinary KIM-1 has been shown to be associated with renal function after transplantation.<sup>10</sup> In addition, levels of LDH significantly increased during brain death compared to the sham group, indicating progressive cell damage. C1-INH reduced LDH levels, although this could not be explained by changes in renal apoptotic in the mitochondria-mediated (intrinsic) pathway. However, the extrinsic pathway of apoptosis and necrosis was not assessed. Treatment with C1-INH did not prevent the increase in infiltrating PMN in the renal cortex. However, information on the dynamics and time frame of the influx of inflammatory cells is absent, since the influx was only determined 4 hours after BD. Previously, significant PMN infiltration has already observed in rat kidneys, 30 minutes after BD.<sup>9</sup> Since, C1-INH treatment was administered 30 minutes after BD, this could explain the ineffectiveness on the influx of PMNs. Unexpectedly, C1-INH treatment led to a significant increase in the influx of M $\Phi$ . However, this increase could be caused by macrophages that decrease inflammation and encourage tissue repair (called M2 macrophages) rather than macrophages that encourage inflammation (called M1 macrophages).

We acknowledge that the present study has limitations. Firstly, despite the significant protection demonstrated by C1-INH in preserving the kidney in our model, further investigations are necessary to evaluate the effect of C1-INH on BD-induced renal injury in larger animals and/

or humans. Secondly, only male rats were used for our experiments. This was due to lack of data on the effect of gender on brain death, as well the reduced ability to activate complement in female rodents.<sup>50,51</sup> In addition, in this study human plasma-derived C1-INH, was used for the treatment of rodents. Furthermore, it is unlikely that monotherapy with C1-INH in the donor would fully abolish the issue of DGF. We hypothesize that the ideal treatment regime in BD would be the combination of hormone replacement therapy, improvement of hemodynamic and immunomodulators. C1-INH treatment could be a key component in this triple combination therapy for BD-induced kidney injury. Lastly, the primary outcome measured was renal injury after BD. The effect of donor treatment with C1-INH on the post-transplantation outcome, therefore, remains to be determined. However, donor treatment is expected to positively impact the post-transplant outcome.

In conclusion, an important finding of this study is that treatment of the donor with a single dose of C1-INH after the induction of BD protects against BD-induced renal injury. These findings open a new window of opportunity for complement inhibition in BD donors to potentially improve kidney grafts for transplantation.

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