Complement modulation to improve donor organ quality

Jager, Neeltina Margaretha

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Complement is activated during normothermic machine perfusion of porcine and human discarded kidneys

In preperation

Neeltina M. Jager
Leonie H. Venema
Henri G. D. Leuvenink

Abstract

Background

The gap between demand and supply of kidneys for transplantation has led to the use of kidneys from marginal donors. Transplantation of marginal donor kidneys is associated with inferior results, reflected by an increased risk of delayed graft function. These inferior results might be explained by the higher immunogenicity of marginal donor kidneys. Normothermic machine perfusion (NMP) could be used to improve the quality of marginal donor kidneys, because NMP provides the opportunity to assess the quality and function of donor kidneys. In addition, the immunological response of marginal donor kidneys could be evaluated during NMP. In this chapter, we evaluated the immunological response during NMP of porcine and human kidneys by measuring complement activation. In addition, we examined the effect of complement activation by the quantification of pro-inflammatory cytokines during NMP of porcine kidneys. Lastly, we assessed the effect of complement activation on renal function during NMP of porcine kidneys.

Methods

Both porcine and discarded human kidneys were normothermic machine perfused for 4-6 hours with a blood-based perfusion solution. Perfusate samples were taken every hour to assess complement activation, the release of pro-inflammatory cytokines and renal function.

Results

Complement activation products, reflected by C3 and C5b-9, were found in perfusate samples taken during NMP of both porcine and human kidneys. Complement perfusate levels were significantly increased during NMP of porcine kidneys. In addition, complement perfusate levels positively correlated with the cytokine perfusate levels of IL-6, IL-8 and TNF-α during NMP of porcine kidneys. Further, porcine kidneys with high C5b-9 perfusate levels had a significant lower creatinine clearance after 4 hours of NMP. In line with these findings, high complement perfusate levels, reflected by C3d/C3 ratio, were seen during NMP of human discarded kidneys. In addition, kidneys retrieved from brain-dead donors had significantly higher complement C3d/C3 perfusate levels during NMP than kidneys retrieved after circulatory death.

Conclusion

Complement perfusate levels are significantly activated during NMP of porcine and human kidneys. Complement activation during NMP of porcine kidneys is positively correlated with the release of cytokines during NMP. In addition, high complement perfusate levels are associated with lower creatinine clearance during NMP of porcine kidneys. During NMP of human kidneys, complement is predominantly activated in kidneys retrieved from brain-dead donors. Therefore, we propose that complement inhibition during NMP might be a promising strategy to reduce renal graft injury and improve graft function prior to transplantation.

Introduction

Normothermic machine perfusion (NMP) is a preservation technique that recently has been reapplied to preserve and assess organ quality prior to transplantation. The idea of *ex vivo* machine perfusion is a far from novel concept. In 1935, Lindbergh and Carrel already developed a device that perfused organs with a steady supply of oxygenated artificial blood.[1] In the subsequent years, NMP disappeared to the background due to the successful introduction of static cold storage (SCS). SCS was cheaper and easier to implement and more importantly it resulted in good transplant outcomes given the quality of the donated kidneys at that time.[2] However, with the increasing gap between demand and supply, the acceptance of kidneys with a lower graft quality was needed. The increasing use of marginal donor kidneys resulted in an increased risk of delayed graft function and inferior renal function compared to standard criteria donor kidneys.[3,4] One of the hypotheses for the inferior results is the higher immunogenicity after transplantation due to elderly donors and increased comorbidities. The increased use of marginal donor kidneys led to the idea of tailoring the preservation method to the renal graft, which resulted in the revival of NMP. During NMP the renal graft is perfused with an oxygenated perfusion solution at 37°C (Figure 1). Maintaining a kidney at a normothermic temperature has many advantages. It provides the possibility of evaluating kidney quality but also to recondition prior to transplantation. Furthermore, it would be possible to characterize immunological responses of donor kidneys. So far, only two studies which showed the potential of NMP to reduce the donor kidney immunogenicity by preventing the activation of the immune system.[5,6]

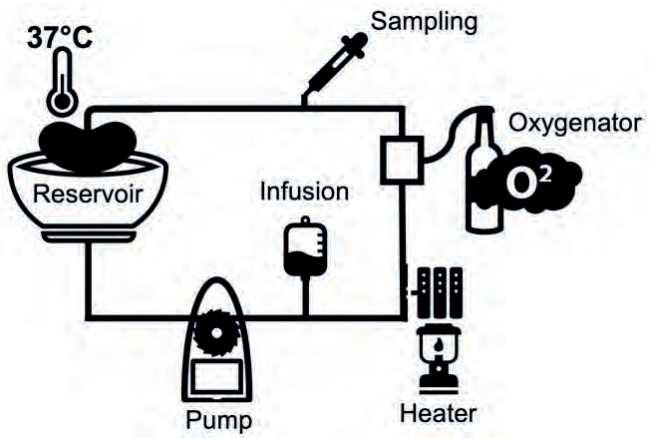


Figure 1. Normothermic machine perfusion set-up.

Kidneys are equipped with a sophisticated localized immune system and previous studies showed that in case of severe injury, such as brain death, the kidney induces a robust local immune response, including activation of the complement system.[5,7] The complement system is part of the innate immune system and consists of more than 30 proteins and can be activated via three pathways: the classical (CP), the lectin (LP) and alternative pathway (AP) that all merge at complement component C3. C3 activation results in the cleavage of C3 into C3a and C3b. In addition, activation of C3 will result in the cleavage of C5 into C5a and C5b-9. C5b-9 is also known as the membrane attack complex (MAC) (Figure 2).[8,9] To avoid inappropriate complement activation under normal conditions, complement is strictly regulated by regulatory proteins. Inappropriate activation of the complement system can have deleterious effects on kidneys and needs to be prevented when possible.[10,11] It is known that the complement system can be activated through contact with foreign surfaces, which is the case during extracorporeal membrane oxygenation (ECMO), cardiopulmonary bypass (CPB) and hemodialysis (HD).[12,13] A NMP system consists of the same components as a CPB and HD system and it is therefore logical to assume that NMP also evokes an inflammatory response. In addition, complement activation also plays a prominent role in renal graft injury, which is induced in both the donor as well as the recipient.[14,15] Given the importance of complement in renal graft injury, modulating the complement system is a potential promising strategy to improve renal transplant outcome.[15,16] However, before we can treat, we first need to get more insight in the role and effects of complement activation during NMP. Therefore, this chapter examined whether complement was activated during NMP of porcine and human discarded kidneys. In addition, the effect of complement activation on the pro-inflammatory response and renal function during NMP of porcine kidneys was investigated.

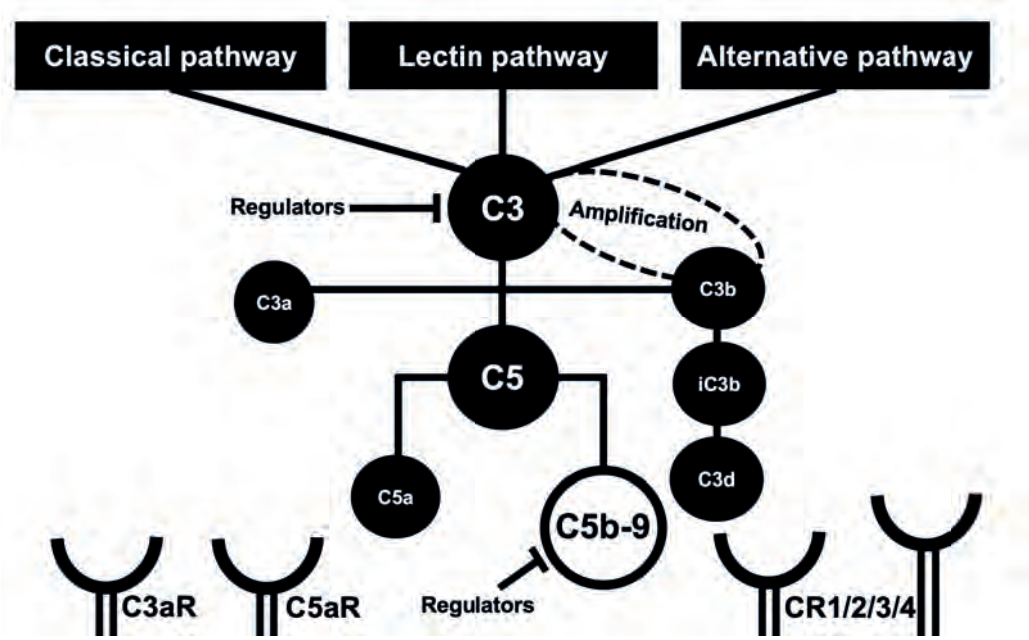


Figure 2. Overview of the complement system.
The complement system is activated via one of the three activation pathways: the classical, the lectin or the alternative pathway. Activation of the pathways leads to cleavage of C3 into C3a and C3b. C3b triggers the amplification loop, which enhances the cleaves of C3. C3b is eventually broken down into iC3b as its primary reaction product, and C3d. Both C3a and C3d are complement activation products, which are specific markers to measure in pigs and human. These breakdown products bind to several receptors including complement receptors (CR) CR1, CR2, CR3 and CR4. Activation of C3 subsequently results in the cleavage of C5 into C5b-9 and C5a. C5b-9 is also called the membrane attack complex (MAC). C5a and C3a are anaphylatoxins, which mediate chemotaxis and thereby produce a pro-inflammatory response.

Material and Methods

Normothermic machine perfusion of porcine kidneys

In the preclinical phase porcine kidneys and autologous heparinized whole blood were used, as described previously.[17] In short, kidneys (n=20) were exposed to a warm ischemia time of 30 minutes to induce ischemic injury. Subsequently kidneys were cold flushed with 180 ml cold (4°C) saline (Baxter BV, Utrecht, The Netherlands) and stored for 24 hours by either static cold storage (SCS) or with hypothermic machine perfusion (HMP) with a mean arterial pressure of 25 mmHg with no active oxygenation or with 21% or 100% oxygen as described earlier.[17] Kidneys of the HMP groups were cannulated for connection to the HMP device (Kidney Assist Transport, Organ Assist, Groningen, The Netherlands) and perfused with 500 ml of University of Wisconsin machine perfusion solution (UW-MP solution, Bridge to Life Ltd., London United Kingdom). SCS preserved kidneys were submerged in 500 ml University of Wisconsin cold storage solution (UW-CS, Bridge to Life Ltd.) and stored on ice. Thereafter, all kidneys were pressure-controlled perfused with a mean arterial pressure of 75 mmHg at a normothermic temperature (37°C) for four hours (Kidney Assist Transport, Organ Assist). The perfusion solution was leukocyte-depleted blood diluted with Ringer's lactate solution and several additives (Table 1). Renal artery and ureter were cannulated with a 12F and 8F cannula respectively. Next, kidneys were flushed with 50 ml cold saline solution to remove remaining UW solution and connected to the NMP device (Figure 1) The perfusion solution was oxygenated via an oxygenator (Medos Medizin AG, Stolberg, Germany) with a mixture of 95% O_2/5% CO_2 at a flow rate of 0.5 L/minute. During NMP there was a continuous supply of nutrients, which was administered at a rate of 20 ml/hour (Table 1). In case the glucose levels dropped below 5 mmol/l glucose 5% was administered.

Table 1.

Composition of the perfusion solution used for normothermic machine perfusion

Perfusion solution - Porcine kidneys	**Perfusion solution - Human discarded kidneys**
500ml Leukocyte-depleted blood	500ml Washed red blood cells
1000mg/200mg Amoxicillin/Clavulanate	1000mg Cefazoline
6mg Mannitol	3mg Mannitol
10ml 8.4% Sodium bicarbonate	10ml 8.4% Sodium bicarbonate
100µl 20mg/ml Sodium Nitroprusside	20ml 10% Calcium Gluconate
300ml Ringer's lactate	500ml NaCl
90mg Creatinine	100ml 20% Albumin
6mg Dexamethasone	
Infusion solution - Porcine kidneys	**Infusion solution - Human discarded kidneys**
80ml 10% Aminoplasmal	80ml 10% Aminoplasmal
17IU Novorapid	17IU Novorapid
	0.5mg Flolan

Normothermic machine perfusion of human discarded kidneys

The kidneys described are included within the Prolonged *ex vivo* normothermic machine perfusion for kidney regeneration (PROPER)-trial registered in the Dutch Trial Register as NL8446. A Dutch initiative from three University transplant centers: Rotterdam Erasmus Medical Center (Erasmus MC), Leiden University Medical Center (LUMC) and the University Medical Center Groningen (UMCG) to introduce and clinically evaluate prolonged NMP. The ten analyzed kidneys were included between the 1st of January 2019 and the 1st of August 2019. The included kidneys were retrieved from donation after brain death (DBD)- and donation after circulatory death (DCD) donors, but discarded postretrieval. Kidneys were cold flushed with University of Wisconsin solution (UW-CS, Bridge to Life Ltd.) and preserved via SCS or with HMP. As a perfusion solution washed red blood cells (RBCs) were used, which were diluted with 0,9% NaCl (Table 1). RBCs were washed with 2L 0.9% NaCl by using a CellSaver (Fresenius C.A.T.S. plus, Fresenius Kabi GmbH, Bad Homburg, Germany). Upon arrival in the UMCG or LUMC, the renal artery and ureter are cannulated with a 12F and 8F cannula respectively. After preparation, the kidney is weighed and flushed with 200ml Ringer's lactate solution and connected to the NMP device (Kidney Assist Transport, Organ Assist). Kidneys were pressure-controlled perfused with a mean arterial pressure of 75 mmHg for six hours. The perfusion solution was oxygenated via a oxygenator (Medos Medizin AG) with a mixture of 95% O_2/5% CO_2 at a flow rate of 0.5 L/minute. During NMP there was a continuous supply of nutrients, which was administered at a rate of 20 ml/hour (Table 1). In case the glucose levels dropped below 5 mmol/l glucose 5% was administered.

Perfusate samples

Perfusate samples during NMP were taken at baseline, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours. Samples at time points 5 hours and 6 hours were only taken during NMP of human discarded kidneys. Samples were collected in EDTA tubes (Biosciences, Plymouth, UK) and stored on ice and centrifuged at 4°C, 2500G for 20 minutes. Plasma was stored at -80°C.

Renal function

Creatinine and sodium concentrations were measured in blood and urine, using routine procedures at the clinical chemistry lab of the University Medical Center Groningen.

Complement assays for perfusate samples after NMP of porcine kidneys

To measure complement activation products in the perfusate samples, complement activation products at the level of C3 were measured. In the perfusate samples retrieved from NMP with porcine kidneys, C3a was measured, which reflects complement activation. C3a was measured with a mouse IgG2bk anti-porcine C3a (clone Z22/8) capture antibody and detected with a mouse-IgG1/k anti-porcine C3a (clone K5/9) detection antibody. A HRP-conjugated secondary goat anti-mouse IgG1 antibody was used as secondary antibody.[18] In addition, C5b-9 was measured as described previously.[19]

In short, the monoclonal antibody aE11, was used as capture antibody and biotinylated monoclonal anti-C6 (clone 9C4) was used for detection. The level was related to the International Complement Standard #2, defined to contain 1.000 complement arbitrary units (AU) per ml.

Complement assays for perfusate samples after NMP of human discarded kidneys

In the perfusate samples from NMP with human discarded kidneys, C3 and C3d were measured and based on these values a C3d/C3 ratio was calculated, which is a measure for complement activation at the level of complement C3. C3d was measured as described previously. Briefly, samples were polyethylene glycol (PEG) precipitated. PEG precipitation is necessary since free C3d shares epitopes with intact C3. All samples were 1:1 diluted with 22% PEG in 0.1Mborate/EDTA buffer (pH 8.32) and incubated on ice for 3 hours. Afterwards they were centrifuged and supernatants were used for C3d quantification. C3d was captured with a monoclonal mouse anti-C3 antibody (sc-28294, Santa Cruz, CA, USA). A rabbit anti-human C3d was used as detection antibody (Dako). Complement C3 was quantified by using a goat anti-human polyclonal C3 capture antibody (Lifespan Biosciences) and a monoclonal mouse anti-human C3 (Lifespan) was used as detection antibody. All samples were measured in duplicates. Values are expressed as ng/ml.

Quantification of cytokines

To detect and quantify pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α), in the perfusate samples we used commercial porcine immunoassay kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Values are expressed in pg/ml.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software v8.3.1. Differences between time points were tested using Kruskal-Wallis test followed by a Mann-Whitney U post-hoc test. Differences between two groups were tested by a Mann-Whitney U test. Spearman correlation coefficients were calculated to determine which cytokines were significantly associated with complement C3a and C5b-9 levels. $P<0.05$ was considered significant. Data are presented as median ± interquartile range (IQR).

Ethics

For the preclinical experiments' slaughterhouse waste material (kidneys and blood) were used. Therefore, no animal ethics committee approval was needed. The clinical study was approved by the local ethical committee and performed in accordance to the principles of the declaration of Helsinki.

Results

Complement is activated during normothermic machine perfusion of porcine kidneys

Complement activation was assessed by the quantification of perfusate levels of C3a and soluble C5b-9 during 4 hours of NMP. First, C3a perfusate levels after 2 and 4 hours of NMP were significantly increased compared to C3a perfusate levels after 30 minutes of NMP. C3a perfusate levels especially seem to increase between 2 and 4 hours of NMP (Figure 3A). Next, complement C5b-9 perfusate levels significantly increased after either 2 and 4 hours of NMP compared to C5b-9 levels after 30 minutes of NMP. In contrast to C3a, the formation of C5b-9 seems to slow down after 4 hours compared to the first 2 hours of NMP (Figure 3B). The preservation methods before NMP did not influence both C3a and C5b-9 perfusate levels during 4 hours of NMP (Supplementary Figure 1A-B).

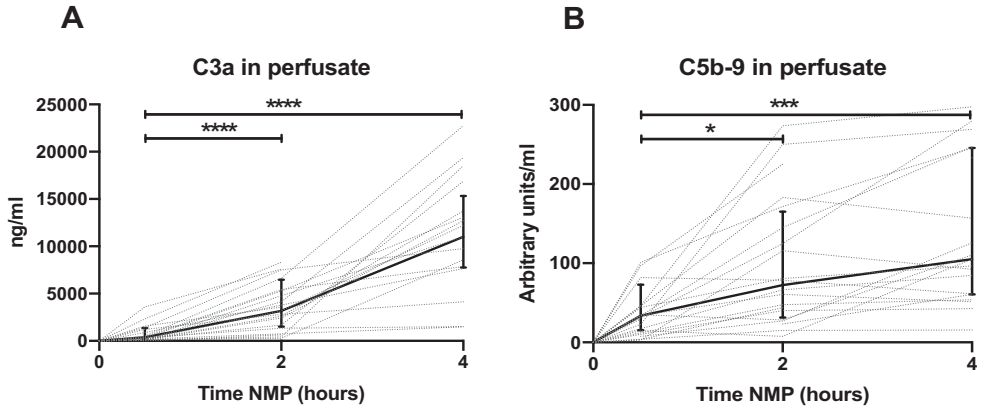


Figure 3. Dynamics of complement perfusate levels during 4 hours of normothermic machine perfusion of porcine kidneys

Dynamics of (A) C3a and (B) C5b-9 perfusate levels during 4 hours of normothermic machine perfusion of porcine kidneys. In perfusate, median perfusate levels of both C3a and C5b-9 increases during normothermic machine perfusion of porcine kidneys. Dotted lines: increase of complement perfusate levels per individual perfused kidney. Solid lines and bars: median ± interquartile range of complement perfusate levels for all kidneys (n=20). *P<0.05, ***P<0.001, ****P<0.0001.

Pro-inflammatory mediators increase during 4 hours of normothermic machine perfusion of porcine kidneys

Perfusate levels of pro-inflammatory cytokines IL-6, IL-8 and TNF-α were quantified during NMP of porcine kidneys. Perfusate levels of IL-6 were significantly increased after 2 and 4 hours compared to baseline levels. Interestingly, IL-6 levels increased exponentially between 2 and 4 hours of NMP (Figure 4A). In addition, perfusate levels of IL-8 were measured, which showed a similar pattern of increase during 4 hours of NMP. In line with IL-6 perfusate levels, IL-8 was exponentially increased between 2 and 4 hours of NMP (Figure 4B). Lastly, TNF-α levels during 4 hours of NMP were examined. TNF-α levels significantly increased after 2 and 4 hours (Figure 4C). Interestingly, the dynamics of TNF-α differs from IL-6 and IL-8. Whereas

IL-6 and IL-8 increase after 2 hours, there was a direct increase in TNF-α visible. Different preservation techniques prior to NMP did not affect cytokine perfusate levels during (Supplementary Figure 2A-C).

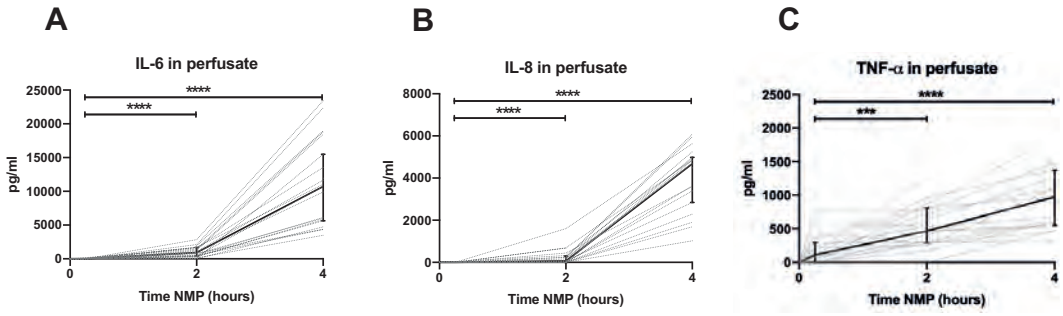


Figure 4. Cytokine perfusate levels during normothermic machine perfusion of porcine kidneys
Dynamics of pro-inflammatory cytokines (A) IL-6, (B) IL-8 and (C) TNF-α in perfusate during normothermic machine perfusion of porcine kidneys. Cytokine perfusate levels significantly increased during 4 hours of normothermic machine perfusion. Dotted lines: increase of cytokine levels in perfusate per individual perfused kidney. Solid lines and bars: median ± interquartile range of cytokine perfusate levels for all kidneys (n=20). ***P<0.001, ****P<0.0001.

Correlation between complement and cytokine perfusate levels during normothermic machine perfusion of porcine kidneys

Next, the correlation between complement perfusate levels and cytokine perfusate levels after 4 hours of NMP of porcine kidneys was examined. Increase in complement C3a perfusate levels strongly correlates with the increase of perfusate levels of IL-6 and IL-8 (Figure 5A-B). In addition, there was a correlation between complement C5b-9 levels and cytokine levels of IL-6, IL-8 and TNF-α after 4 hours of NMP, but to a lesser extent (Table 2).

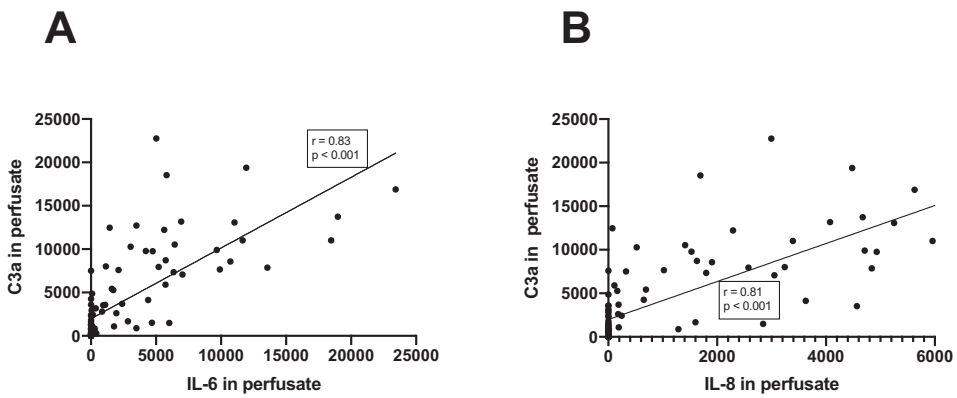


Figure 5. Correlation between complement C3a levels and cytokine levels after 4 hours of normothermic machine perfusion

Correlation between (A) complement C3a and IL-6 levels in perfusate and (B) correlation between complement C3a and IL-8 levels in perfusate after 4 hours of NMP of porcine kidneys. Spearman's correlation coefficient (r) and p-values are indicated.

Table 2.

Correlation between complement perfusate levels and cytokine perfusate levels after 4 hours of normothermic machine perfusion of porcine kidneys

Perfusate levels	Correlation with C3a		Correlation with C5b-9	
	r	P-value	r	P-value
IL-6	0.83	<0.0001	0.52	<0.0001
IL-8	0.81	<0.0001	0.48	<0.0001
TNF-alpha	0.66	<0.0001	0.54	<0.0001

Abbreviations: r, correlation coefficient.

Kidneys with high C5b-9 perfusate levels after 4 hours of NMP have a significant lower creatinine clearance

Next, subgroups analysis was performed for C5b-9 perfusate levels after 4 hours of NMP of porcine kidneys (Figure 3B). Two subgroups (n=10/group) were formed based on the median value of C5b-9 after 4 hours of NMP (median 105 AU/ml) and the creatinine clearance for these two subgroups were calculated. Kidneys with C5b-9 perfusate levels above 105 AU/ml after 4 hours of NMP had a significant lower creatinine clearance than kidneys with C5b-9 levels below the median value (Figure 6).

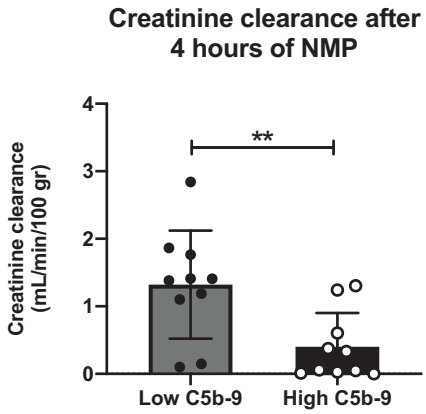


Figure 6. Creatinine clearance after 4 hours of normothermic machine perfusion of kidneys with low *versus* high C5b-9 perfusate levels.
Kidneys (n=10) with low C5b-9 perfusate levels after 4 hours of NMP had a significant higher creatinine clearance than kidneys (n=10) with high C5b-9 perfusate levels after 4 hours of NMP. Subgroups were based on the median C5b-9 perfusate level after 4 hours of NMP, which was 105 AU/ml. **p<0.01.

Complement is activated during 6 hours of normothermic machine perfusion of human discarded kidneys

To translate the experimental results seen during NMP of porcine kidneys, complement activation during NMP of human discarded kidneys was investigated. These human discarded kidneys were retrieved from DBD and DCD donors. Baseline characteristics of the perfused kidneys are shown in Table 3. To investigate whether complement was activated during NMP of human discarded kidneys, complement C3 and C3d perfusate levels were measured. Complement C3 perfusate levels did not change during 6 hours of NMP (Figure 7A). In addition, C3d perfusate levels were measured, C3d perfusate levels did significantly increase during 6 hours of NMP, with the biggest increase between 4-6 hours of NMP (Figure 7B). However, complement C3d levels depend on the concentration of complement C3. So to measure complement C3d activation independent from variations in C3 complement levels, complement activation was measured by calculating the C3d/C3 ratio (Figure 7C). The C3d/C3 ratio significantly increased after 6 hours of NMP compared to baseline levels (30 minutes after NMP).

Table 3.

Baseline characteristics of human discarded kidneys perfused during six hours of normothermic machine perfusion

Donor age (years)	66 (54-74)
Female donor (n; %)	1; 10
Reason for decline (n; %)	
- **Artheriosclerosis**	2; 20
- **High donor age**	1; 10
- **Dissection renal artery**	1; 10
- **High transaminase levels**	1; 10
- **Suspicious for malignancy**	1; 10
- **Medical reasons (not further specified)**	4; 40
Donor type (n; %)	
- **DBD donor**	5; 50
- **DCD donor**	5; 50
Organ preservation method (n; %)	
- **Static cold storage**	4; 40
- **Hypothermic machine perfusion**	6; 60
Cold ischemia time (n; %)	
- **< 15 hours**	4; 40
- **> 15 hours**	6; 60

Abbreviations: DBD, donation after brain death; DCD, donation after circulatory death.

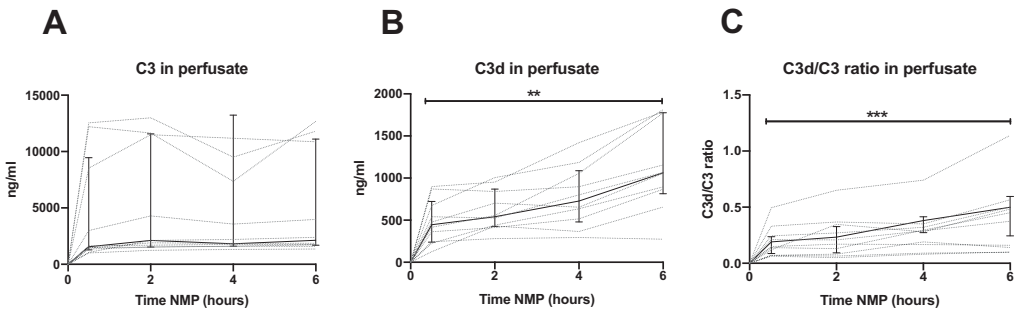


Figure 7. Complement perfusate levels during normothermic machine perfusion of human discarded kidneys.

(A) Complement C3 (in ng/ml) and (B) C3d levels (in ng/ml) and (C) the calculated C3d/C3 ratio in perfusate during 6 hours of normothermic machine perfusion of human discarded kidneys. Dotted lines: increase of complement per individual kidney. Solid lines and bars: median ± interquartile range for all kidneys (n=10). **P<0.01, ***P<0.001.

Kidneys retrieved from brain-dead donors have significant higher complement perfusate levels after 6 hours of normothermic machine perfusion

Lastly, perfusate C3d/C3 ratio during NMP of kidneys retrieved from DBD *versus* DCD donors were calculated. Kidneys retrieved from DBD donors had a significant higher C3d/C3 ratio at 6 hours of NMP than kidneys retrieved from DCD donors (Figure 8). During NMP of kidneys retrieved from DBD donors, there was a significant increase of the C3d/C3 ratio over time. In contrast, C3d/C3 ratio during NMP of kidneys retrieved from DCD donors did not significantly change over time. No differences in C3d/C3 ratio were seen based on different preservation methods (SCS *versus* HMP) or based on the cold ischemia time (shorter *versus* longer than 15 hours) (data are not shown).

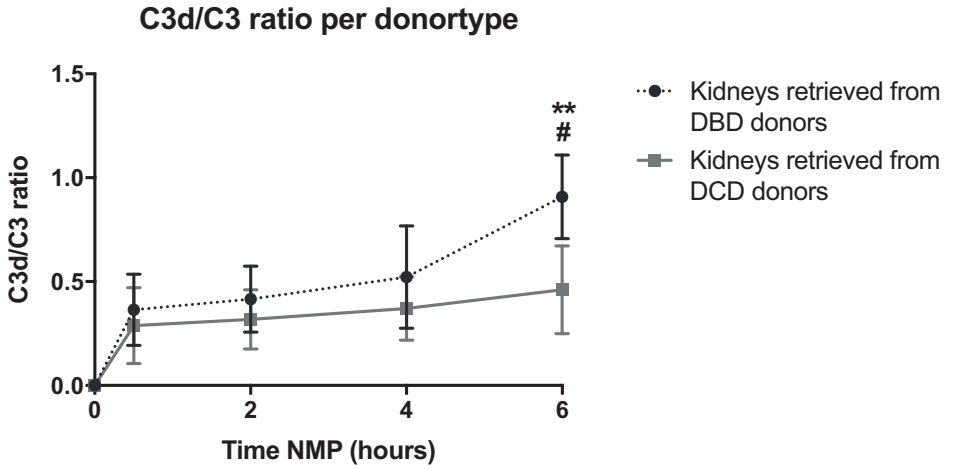


Figure 8. Perfusate C3d/C3 ratio of kidneys retrieved from brain-dead donors are significantly higher after 6 hours of normothermic machine perfusion.

The differences in C3d/C3 ratio during normothermic machine perfusion based on the type of donor. Kidneys retrieved from brain-dead donors have a significant higher C3d/C3 ratio after 6 hours of normothermic machine perfusion than kidneys retrieved after circulatory death. Data is shown as median ± interquartile range. The asterisks denote a significant difference between baseline C3d/C3 ratio and C3d/C3 ratio after 6 hours of NMP of kidneys from brain-dead donors. The hashtag denotes a significant difference in C3d/C3 ratio between the two groups, kidneys retrieved from DBD and DCD donors. #P<0.05, **P<0.01. Abbreviations: DBD, donation after brain death; DCD, donation after circulatory death.

Discussion

In this chapter complement activation during NMP was assessed by using porcine and human discarded kidneys. This chapter demonstrates that complement is activated during NMP of porcine kidneys, as reflected by the presence of complement activation fragments in the perfusate. Further, complement perfusate levels during NMP of porcine kidneys positively correlate with cytokine perfusate levels. Porcine kidneys with high C5b-9 perfusate levels after 4 hours of NMP have a significant lower creatinine clearance than kidneys with low C5b-9 perfusate levels. In line with these findings, complement is significantly activated during NMP of human discarded kidneys. Kidneys retrieved from brain-dead donors have significantly higher complement perfusate levels after 6 hours of NMP than kidneys retrieved after circulatory death.

Looking at the dynamics of complement activation in NMP, complement is immediately activated at the start of NMP, given the evident increase within the first 30 minutes. Based on other *ex vivo* set-ups our results are in line with studies describing complement activation in ECMO, CPB and HD.[20,21] All these studies reveal an rapid increase of complement C3 and C5b-9 within the first 15 minutes.[22,23] The rapid increase in complement activation products could be due to the initial blood-to-material contact, already described a decade ago.[20,24] The contact of blood with the artificial surface results in an immediate adsorption of serum proteins, i.e. complement C3 and immunoglobulin G, which results in the activation of the complement system. After the initial activation, complement perfusate levels continue to rise up to the end of NMP. We speculate that this is due to the absence of a negative feedback loop. *In vivo* complement activation is regulated via plasma and membrane-bound regulators, which avoid inappropriate complement activation.[25] However, complement activation is not regulated in absence of these regulators during *ex vivo* machine perfusion.

The consequences of complement activation during NMP are unknown. This chapter shows that complement activation is strongly correlated with the release of pro-inflammatory cytokines IL-6, IL-8 and TNF-α. Our results are in line with previous results, demonstrating the release of pro-inflammatory cytokines IL-1ß, IL-6 and IL-18.[26] Interestingly, the dynamics of IL-6 and IL-8 differ from TNF-α. TNF-α perfusate levels increase from the start of NMP, while IL-6 and IL-8 perfusate levels start to increase after 2 hours of NMP. This suggest that IL-6 and IL-8 might be produced via a TNF-α dependent pathway.[27] Together, these cytokines might propagate further release of adhesion molecules and polymorphonuclear cells contributing to a pro-inflammatory state.[28] Therefore, the release of these pro-inflammatory cytokines might cause tissue damage.

So far, the consequences of the inflammatory response seen during NMP on renal graft function remains unknown.[29] Activation of complement during NMP might be beneficial, because it could exhaust the complement activation capacity of the renal graft prior to ischemia reperfusion in the recipient. By doing so, less complement is activated, including the subsequent release of pro-inflammatory cytokines. Altogether, this might attenuate the inflammatory response seen during ischemia reperfusion in the recipient. However, complement activation during NMP seems undesirable. Complement activation in other

phases of transplantation, i.e. brain-dead donors and during ischemia and reperfusion, resulted in renal tubular injury causing renal graft dysfunction.[15] In accordance, this chapter showed that porcine kidneys with high C5b-9 perfusate levels during NMP have a significant lower creatinine clearance than porcine kidneys with low C5b-9 perfusate levels at the end of NMP. This could be explained by previous findings which suggest that C5b-9 is essential in the induction of renal injury following ischemia reperfusion.[30]

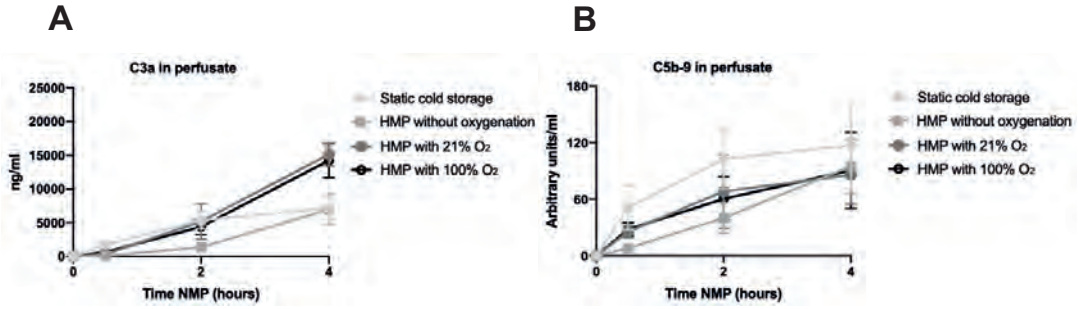
A potential strategy to attenuate complement-mediated renal injury during NMP might be the use of a complement inhibitor. One of the proposed mechanisms of complement activation in NMP is AP activation by binding of properdin or C3b to the tubes or oxygenator membrane of the *ex vivo* circuit. Another proposed mechanism, which is previously suggested as a route of complement activation in HD, is activation via the LP.[12] Therefore we propose, that during NMP, the LP can be activated by the binding of ficolin-2 to the membrane of the oxygenator. Before targeting early complement components during NMP, profound knowledge about the underlying pathophysiology and which complement (activation) pathways are involved is necessary. Nevertheless, modulating the complement system during NMP seems to be a promising strategy.

In the view of a potential treatment strategy, we measured complement activation levels during NMP of human discarded kidneys. Similar to NMP of porcine kidneys, complement activation levels are significantly increased during NMP of human kidneys, as reflected by the increased C3d/C3 ratio after 6 hours of NMP. In addition, we demonstrate that kidneys retrieved from brain-dead donors have a significant higher C3d/C3 ratio than kidneys retrieved after circulatory death. This might be due to the increased immunogenicity of brain-dead donors compared to DCD donors. Brain death itself results in the activation of the immune system, which results in both systemic and local inflammation.[31] Therefore, we postulate that kidneys retrieved from brain-dead donors are more likely to provoke and activate an inflammatory response during NMP.[15,16,32] Looking forward, kidneys retrieved from brain-dead donors might benefit more from treatment with a complement inhibitor during NMP than a kidney retrieved after circulatory death. Kidneys retrieved from DCD donors showed a peak in complement activation during NMP in the first 30 minutes, after which no further increase was seen. So, complement activation during NMP seems to occur predominantly in kidneys retrieved from brain-dead donors. No differences in complement activation during NMP were seen between preservation with SCS or HMP. Remarkable, because HMP is superior to SCS in deceased donor renal transplantation, reflected by the lower incidence of delayed graft function after HMP.[33] We speculate that we do not see any differences between complement activation in HMP *versus* SCS, due to the fact that complement is not activated or to a lesser extent at low temperatures. This is confirmed in a pilot study performed by our research group, no complement activation fragments were measured in perfusate samples taken during HMP (data not shown). In accordance, multiple studies describe low or no complement activation under other hypothermic conditions.[34,35]

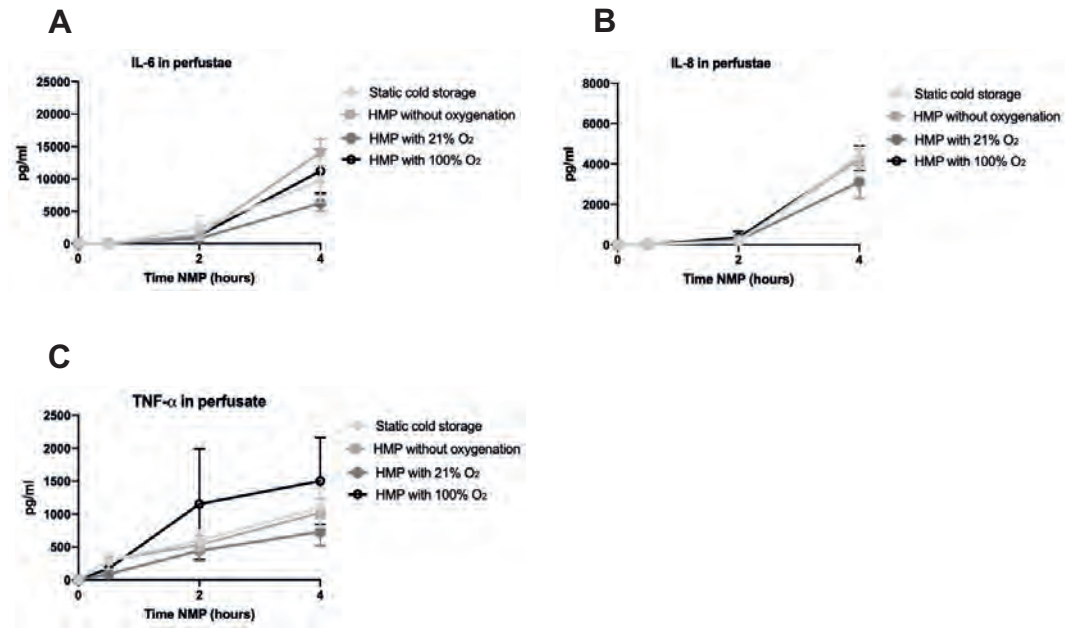
Looking at the current results, this chapter has some limitations. First, the number of kidneys included in the human cohort is small and therefore might impact the statistical analyses. In addition, we did not correlate complement activation during NMP of human kidneys to renal injury or renal function. However, we achieved to proof that complement is activated during NMP of human kidneys and it is of interest to perform further research. Therefore, further research needs: (i) to examine the functional consequences of complement activation during NMP of human kidneys, (ii) to investigate whether complement is locally activation during NMP and (iii) to test the efficacy of a complement inhibitor during NMP.

In conclusion, this chapter shows that complement is activated during NMP of both porcine and human kidneys. Complement activation during NMP of porcine kidneys is positively correlated with the increase of cytokine perfusate levels during NMP. In addition, porcine kidneys with high C5b-9 perfusate levels after 4 hours of NMP have a significant lower creatinine clearance. In line with these findings, during NMP of human kidneys complement is significantly activated. Complement is predominantly activated during NMP of kidneys retrieved from brain-dead donors. Therefore, we propose that complement inhibition during NMP might be a promising strategy to reduce renal injury and improve renal graft function prior to transplantation.

Supplementary Figures



Supplementary Figure 1. C3a and C5b-9 perfusate levels for the different preservation groups.
(A) C3a and (B) Cb5-9 perfusate levels during 4 hours of NMP of porcine kidneys. C3a and C5b-9 perfusate levels are visualized for the different preservation groups: static cold storage, hypothermic machine perfusion (HMP) without oxygenation, HMP with 21% oxygen and HMP with 100% oxygen. N=6 per group. Data are shown as median ± interquartile range. Abbreviations: HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.



Supplementary Figure 2. Cytokine perfusate levels for the different preservation groups.
(A) IL-6, (B) IL-8 and (C) TNF-α perfusate levels during 4 hours of NMP of porcine kidneys. IL-6, IL-8 and TNF-α perfusate levels are visualized for the different preservation groups: static cold storage, hypothermic machine perfusion (HMP) without oxygenation, HMP with 21% oxygen and HMP with 100% oxygen. N=6 per group. Data are shown as median ± interquartile range. Abbreviations: HMP, hypothermic machine perfusion; NMP, normothermic machine perfusion.

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