Modelling acute antibody-mediated rejection of human kidney transplants using ex-vivo warm machine perfusion

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Summary

Background Transplant rejection is a major cause of graft loss and morbidity. Currently, no human models of antibody-mediated rejection (AMR) exist, limiting mechanistic investigation and organ-specific targeted therapy. Here, using 12 human kidneys and ex-vivo normothermic machine perfusion, we demonstrate phenotypes of AMR after addition of antibodies against either human HLA class I or blood group antigens (A, B), thus modelling clinical AMR that can follow HLA incompatible (HLAi) or blood group incompatible (ABOi) transplantation.

Methods Discarded human kidneys with wide ranging demographics and cold ischaemia times (11–54 h) were perfused with red blood cells and fresh frozen plasma (FFP) as a source of complement/coagulation factors. For the HLAi model, 600 μg of W6/32 anti-class 1 HLA antibody was added to the circuit (time '0'). For the ABOi model, high titre FFP of the relevant blood group antibody was added. Renal blood flow index (RBFi, mL/min/100 g), C3 desArg, prothrombin fragments 1 + 2 and histology were determined. Our endpoints included haemodynamic changes, thrombosis, and biopsy proven complement deposition.

Findings Compared to control kidneys perfused without anti-donor antibodies, both models demonstrated haemodynamic collapse after antibody perfusion with only the HLAi model showing glomerular C4d deposition.

Interpretation We show that a clinically relevant human kidney model of AMR is feasible, and anticipate that these models, with refinements, could provide a basis to test different strategies to prevent AMR.

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Introduction

Antibody-mediated rejection (AMR) is a common and potentially devasting sequela of renal allograft transplantation which, despite immunosuppressive therapy, may result in up to 30% of some patient cohorts experiencing graft loss within one year post-

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Research in context

Evidence before this study

Antibody-mediated rejection (AMR) is a devasting complication following organ transplantation that can result in graft loss and much morbidity. Current management paradigms rely on systemic treatment which may pose the risk of overwhelming sepsis or even mortality. Targeted organ therapy against AMR has been proposed as an alternative management strategy in transgenic and preclinical animal models but these have inherent limitations with respect to direct clinical applicability. Currently there are no human kidney models of AMR with translational relevance that may be used to study rejection mechanisms and investigate organ specific targeted therapy.

Added value

We present a clinically relevant, reproducible, and translational human kidney model of antibody-mediated rejection using human kidneys and warm machine perfusion technology (ex-vivo normothermic perfusion).

Implications of all available evidence

This human transplant model may provide a relevant clinical platform to help study and improve our insight and understanding of transplant rejection mechanisms and accommodation. More importantly, it may facilitate and strengthen the testing of targeted organ therapeutic approaches against AMR, with direct applicability to other organ transplant types.

transplantation.[1,2] Both blood group and HLA antibodies can cause AMR, characterized by a complex molecular and cellular interplay at the kidney endothelial cell surface. AMR involves complement activation, coagulopathy, endothelial cell damage, recruitment of leucocytes, monocytes and natural killer cells and vascular damage causing a spectrum of injury phenotypes within the allograft. AMR may be hyperacute (occurring within the first 24 h), acute (within weeks) or chronic (months to years). Hyperacute rejection associated with pre-formed donor specific antibody (DSA), is categorized by catastrophic graft vascular thrombosis, haemorrhage and necrosis as the complement and coagulation cascades are irreversibly activated by antibody binding to target antigens on graft endothelial cells.[3] This is conventionally avoided by performing blood group compatible and crossmatch negative transplants.

Patients may, however, become HLA allosensitised after exposure to previous solid organ transplants, blood transfusions, pregnancies, coronary bypass allografting and tissue grafting.[4,5] Approximately 30%–40% of patients on the kidney transplant waiting list have HLA antibodies capable of binding a wide variety of donor HLA, making it very difficult to match them to any potential deceased donor.[6] These patients are conventionally considered highly sensitised if their antibodies bind ≥85% of the organ donor pool. The incidence of de-novo DSA in previously non-sensitised renal recipients has been reported as 7% by 5 years post transplantation, 20% by 10 years and up to 40% in children.[7] The presence of these antibodies is not only a risk factor for AMR in renal transplant recipients who receive an incompatible organ, but also a predictor of graft failure and injury.[8–11] Furthermore, de-novo DSA AMR has been shown to be an independent risk factor for allograft loss.[12]

Direct HLA incompatible (HLAi) or ABO incompatible (ABOi) transplantation may be offered to highly sensitised patients or those with an ABO-incompatible living donor.[13,14] This involves removing anti-blood group antibodies and/or HLA antibodies from the recipient, prior to transplantation—effectively "desensitizing" the patient to allow safe transplantation to take place. Typically, this is feasible only in patients with lower titres of anti-donor antibodies. Conventional protocols involve a combination of intravenous immunoglobulin therapy with several episodes of plasmapheresis and/or immunoadsorption, sometimes in association with B-cell depleting therapy. More enhanced induction and maintenance immunosuppression is also used and future protocols may utilise novel therapies such as *Imlifidase*[15,16] or neonatal FcR inhibition.[17] If desensitisation is successful and transplantation proceeds, recipients still may have an increased risk of severe post-operative AMR (which could be as early as within 14 days post transplantation), bleeding and mortality.[18–21]

There is therefore a need to explore novel paradigms in antibody-incompatible transplantation which, despite becoming a clinical reality, still pose challenges with respect to optimizing graft survival, reducing early aggressive rejection and infection and giving access to patients deemed potentially un-transplantable (or at the least, very high risk) due to high levels of antibody.[22–24] The endothelium provides a platform, not only for antibody binding and complement activation but also one that confers resistance to injury. Hence, targeted therapeutic manipulation of the endothelium to help increase this resistance and upregulate expression of protective complement regulatory and coagulation regulatory proteins may help induce "accommodation" in the graft by protecting against the deleterious effects of antibody-mediated injury. These strategies have been tested successfully using transgenic or pre-clinical models of AMR,[25–27] but currently there is no approach to readily facilitate testing such strategies in human models with direct translational relevance. Following on from previous work in a porcine hypothermic perfusion

model[28] we report the development of a translational human model of AMR model by determining how human kidneys respond to both HLA and anti-blood group antibodies whist undergoing ex-vivo normothermic machine perfusion (EVNP) with blood-based solutions and fresh frozen plasma as a source of complement and coagulation factors.

EVNP is a perfusion technique based on paediatric cardiopulmonary bypass technology offering organ physiology preservation, viability assessment and monitoring in real time. The technique involves perfusing an organ with warm oxygenated plasma-free red blood cell-based solution and has been used in clinical transplantation to resuscitate and assess organs deemed initially unsuitable for implantation.[29–33] Hence using clinical grade EVNP technology allows the opportunity to create an experimental AMR model perfusion system that could have potential translational capability for investigating and testing of organ specific strategies to prevent rejection.

Methods

Ethics and study approvals

This study involved controlled laboratory experiments on discarded human kidneys offered for research. Permissions were obtained from NHS Blood and Transplant (NHSBT) and our local Renal Project Board to use discarded human organs. Additionally, permission was obtained from our local hospital blood bank to use discarded packed red cell units and fresh frozen plasma. All organs were accepted under approved consent pathways (NHSBT) and no further Research and Ethics approvals (REC) were required. Exclusion criteria for accepting organs were severe organ parenchymal/vascular retrieval damage, very poor perfusion at retrieval after cold flush with evidence of possible intrarenal thrombosis, high resistance flush on the back table prior to EVNP, kidneys that appeared mottled with parenchymal petechiae over its surface (implying possible microthrombi), and the presence of multiple vessels that could not all be cannulated. The project was approved and registered by Guy's and St Thomas' Trust Research and Development (Study registration RJ115/N033). All accepted kidneys were delivered to Guy's hospital via transport services.

EVNP technique

The EVNP technique for our study is identical to previously established clinical EVNP protocols[34] with the exception of two refinements. Conventionally, the clinical EVNP circuit has 3000 units of heparin within its circulating volume,[29] however, this dose would not be appropriate for an experimental AMR model where thrombosis would be an expected endpoint. The EVNP circuit needed to be anticoagulated to prevent clotting in the oxygenator and tubing which will occur irrespective of AMR. We therefore needed to achieve a critical balance between maintaining circuit flow whilst permitting activation coagulation and thrombosis in experimental kidneys to occur in the presence of antibody. Hence, we determined, using a dilution heparin clotting assay that we could reduce the heparin dose to an acceptable minimum level of 375 units that would allow thrombosis to occur in a rejection model yet maintain circuit flow (Supplementary S1). We also used clinically discarded fresh frozen plasma (FFP) as a source of complement and coagulation factors for all three groups. We determined the titre of anti-A and/or anti-B antibodies present in the FFP, using a haem-agglutination test against standardised red blood cells of known blood group (the titre being the number of serial dilutions of FFP at which hemagglutination ability disappears). Each FFP bag was subsequently stored and labelled with the relevant titre value (Supplementary S2).

All kidneys in our study were surgically prepared on the back bench table with cannulation of the renal vessels. The EVNP circuit (Fig. 1) was primed with 250 mL Ringer's solution and one unit of ABO-cross-matched packed red cells and 375 units of heparin. Standard infusions were added to the venous reservoir as described previously and the perfusate circulated to reach steady-state temperature and oxygenation.[30] The kidney was then placed in a stainless-steel chamber and perfusion commenced. During perfusion we monitored renal blood flow (RBF) at 5-min intervals and determined the Renal Blood Flow Index (RBFi mL/min/100 g) in addition to observing the macroscopic appearance of the kidney. Renal blood flow index was calculated using the weight of the kidney prior to cannulation. All kidneys underwent standard EVNP to determine prior suitability for experimental intervention as defined by achieving steady state with respect to a stable (or rising) renal blood flow index over at least a 15-min period. Once achieved, the experimental intervention was introduced (see below for details of the separate model systems). Samples for C3 desArg levels and Prothrombin fragments 1 + 2 were obtained both pre-and post-intervention of W6/32 antibody, high titre FFP or low titre FFP for the HLAi, ABOi and control groups, respectively. These samples were subsequently centrifuged and stored at –80 °C for complement and coagulation analysis (Supplementary S3). All perfusion experiments were concluded at a maximum of 2 h post-experimental intervention. Multiple renal tissue biopsies were taken for frozen sections to enable immunofluorescence analysis (C4d deposition) and conventional paraffin sections to determine the degree of injury (Supplementary S4). All organs and consumables were disposed of according to Human Tissue Authority (HTA) and local hospital and laboratory regulations. All assessments were correlated with the

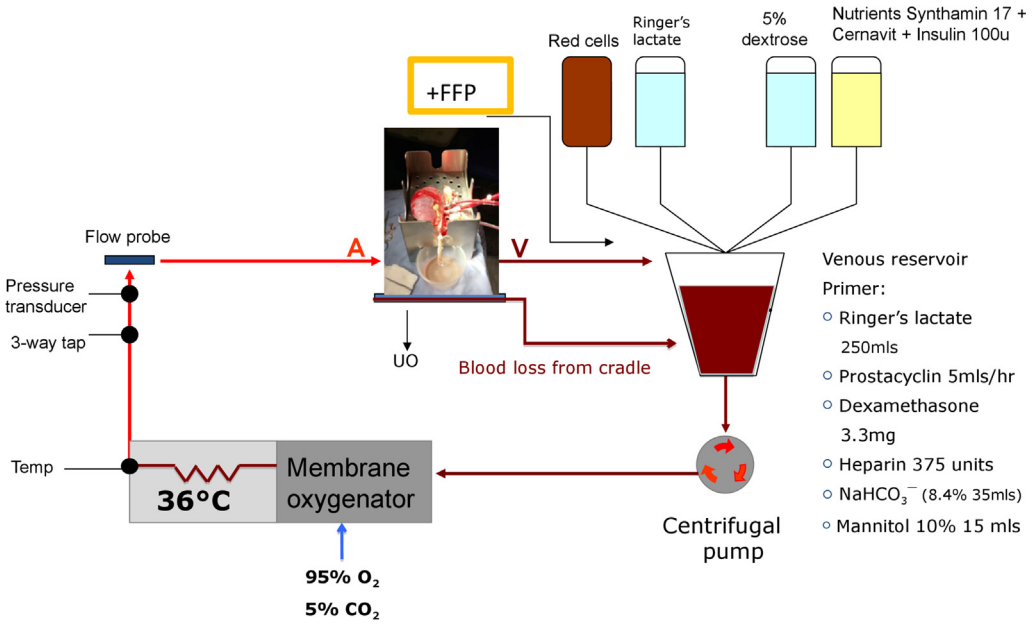

***Fig. 1:* EVNP circuit infusions adapted for the experimental AMR model.** FFP = Fresh Frozen Plasma; UO = urine output; A = arterial inflow; V = venous outflow).

haemodynamic characteristics to build up a phenotype of AMR in the two model systems.

With respect to the experiments, we randomised at every opportunity when it was possible, for instance when we were allocated two kidneys from the same donor; in these circumstances we randomly allocated each kidney to either control perfusion or addition of antibody, and to whether the control perfusion should be done first or second. However, mostly we were allocated single kidneys and we prioritised control perfusions until we had acquired appropriate numbers, before starting to add the anti-class I antibody. For the ABOi experiments, we were constrained by the blood group of the donor and the availability of FFP with appropriate titre of anti-A or anti-B; when the 'right' combinations were available, we prioritised ABOi experiments.

HLAi AMR model

As a source of antibody for the HLAi AMR model, we used commercially available mouse anti-human class I monoclonal W6/32 (ab23755 Mouse monoclonal [W6/32]) to HLA Class I with low endotoxin and Azide free (*Abcam Company, UK, RRID: AB447659*). We previously determined the dose of W6/32 (600 ug) that had the ability to fix complement in clinically discarded human FFP and cause cell lysis using a complement dependent cytotoxicity (CDC) test with human blood lymphocytes (Supplementary S5). For this model, we used blood group O offered kidneys (which could not be used in the ABOi model as they lack expression of A or B antigens) and FFP units with low titres of anti-A or anti-B antibody (<1:8 level in circuit). After demonstrating that kidneys reached stabilization during EVNP, FFP was added to the circuit with 600 ug of W6/32 antibody and we maintained observations as described above.

ABOi AMR model

Blood group A and B kidneys were used in this model, with high titre anti-A or anti-B FFP from an appropriate blood group (Table 1). These titres were determined using the same method in Supplementary S2. The FFP needed to contain sufficient concentration of antibody defined as being able to generate >1:8 titre after dilution in the EVNP circuit (as this is the clinically relevant threshold to perform ABOi transplants.[13] This meant choosing an FFP bag with a minimum titre concentration of 1:64, as this would be further reduced by dilution to approximately 1:16–32 caused by the EVNP circuit volume. As in the HLAi model, after demonstrating that kidneys reached RBFi stabilisation, we added the appropriate unit of FFP and maintained observations as described above.

Controls

Control experiments were performed by adding only low titre FFP bags (<1:8) after kidneys had reached the accepted criteria for intervention. The kidneys were

Discarded kidney group offered	FFP Group for use experimentally for discarded kidney	Antibodies within the FFP	Packed cells RBSs for use experimentally for discarded kidney
A or B or AB	O	Anti-A and Anti-B	O
B or AB	A or O	Anti-B	A/O
A or AB	B or O	Anti-A	B/O

Table 1: **This shows the blood group of the kidney and the possible combinations of FFP/packed red cells that can be used with a particular blood group kidney for the ABOi model.**

monitored, and parameters measured in the same manner as the experimental kidneys above.

Statistics

All data were tested for normality using the Shapiro–Wilk test. Differences in demographic data between ABOi, HLAi and control groups were examined using the Kruskal–Wallis test or the Chi-squared test. Two-sided tests were conducted and $p < 0.05$ was considered statistically significant. Data were analysed using IBM SPSS Statistics for Macintosh version 25 (IBM, Armonk, NY, USA).

Role of funders

The funding sources did not have any role in the study design, methodology, data collection and analyses, manuscript writing or any decisions to submit the work for publication.

Results

Establishing a human kidney model of AMR

We compared perfusion of human kidneys with packed red cells and FFP in the presence of either a monoclonal antibody against HLA class I (to mimic HLAi) or anti-A or anti-B antibodies (to mimic ABOi) with that of a control group. Twelve human kidneys were offered for research; all had been discarded for clinical transplantation for various reasons including, donor age, prolonged cold ischaemia times, presence of malignancy in the donor kidney or other viscera or poor in situ cold perfusion flush at the time of retrieval. The blood group type of FFP and packed red cell unit to be used in each model setting based on the blood group type of the kidney is shown in Table 2. There was no significant difference in median donor age ($p = 0.89$), donor gender ($p = 0.05$), donor type ($p = 0.89$) and cold ischaemia times ($p = 0.15$) between the three groups (Table 3). All kidneys in the three groups underwent initial warm perfusion to achieve a stabilisation phase with respect to renal blood flow index (RBFi, mL/min/100 g) prior to proceeding with the experimental phase. This stabilisation phase was critical as it allowed for kidneys from donors with different demographics and cold ischaemia times to reach a suitable physiological standard for experimental intervention. This was defined as a steady (no more than 5 mL variation) or improving RBFi over a 15-min period (recorded at 5-min intervals). This would typically occur after 60 min of warm perfusion (after initial ischaemia-reperfusion injury) but could take longer to achieve. In addition, the RBFi of at least >20 mL/min/100 g at the point of experimental intervention was required as a kidney may have a stable, albeit low renal blood flow state that would not allow any dynamic changes in renal blood flow to be ascertained after experimental intervention. The criteria for stabilisation were predetermined by our initial EVNP studies with human kidneys (Supplementary S6).

Control group with no HLA nor (high titre) blood group antibodies

Fig. 2 demonstrates the RBFi against perfusion time for the control kidneys. All these kidneys had low antibody titre level FFP added (<1:4 in circuit) after achieving prior RBFi stabilisation. The haemodynamic parameters after addition of FFP do not demonstrate any significant RBFi changes. $K5_{Cont}$ demonstrated the least variation in RBFi upon adding FFP. Of note, $K3_{Cont}$ suffered significant blood loss (150–200 mls) immediately upon starting warm perfusion from unidentified branches of the main renal artery that were not ligated. The kidney suffered continuous bleeding throughout the perfusion cycle with low volumes in the venous reservoir which contributed to the declining flow in this organ and this experiment was abandoned approximately 1 h after adding FFP. $K2_{Cont}$ did demonstrate a gradual decline in RBFi after 1 h 15 min of perfusion whereas K4Cont had a fall in RBFi that subsequently recovered after 45 min of perfusion. All kidneys in this group maintained a pink and well-perfused macroscopic appearance throughout their perfusion cycle. C4d immunostaining was negative for all kidneys in this group and histology demonstrated the expected donor vasculopathy with moderate to severe acute tubular injury in all kidneys in this group with no evidence of any microthrombi.

HLAi group with W6/32 anti-class 1 antibody

All three kidneys ($K1_{HLAi}$ – $K3_{HLAi}$) in the HLAi experimental kidneys were blood group O (Table 2). All kidneys in this group reached stability with respect to Renal Blood Flow Index (RBFi) prior to intervention with low titre FFP (<1:8 in circuit) and (pre-determined)

Kidney number and model tested	Donor type	Donor age (yrs)/sex/blood group	CIT hrs: min	Blood group type of PRBC's/FFP used in circuit with initial antibody titre value
$K1_{HLAi}$	DCD	47 M; O +ve	15:29	O/B (anti A 1:4)
$K2_{HLAi}$	DBD	78 F, O +ve	10:50	O/B (anti A 1:8)
$K3_{HLAi}$	DCD	67 M, O +ve	26	A/O (neat)
$K1_{ABOi}$	DBD	64 F, A +ve	47:40	A/O (anti A 1:128; anti B 1:64
$K2_{ABOi}$	DCD	57 F, A +ve	33:55	O/O (anti A 1:256, anti B 1:128
$K3_{ABOi}$	DCD	71 F, A +ve	17:15	O/O (Anti A 1:128: anti B 1:16)
$K4_{ABOi}$	DCD	63 F, B +ve	32:3	O/O (Anti A 1:256: anti B 1:512)
$K1_{Cont}$	DCD	47 M; O +ve	19:44	O/B (anti A 1:4)
$K2_{Cont}$	DBD	64 F, A +ve	53:50	A/A (anti B 1:32)
$K3_{Cont}$	DCD	67 M, O +ve	30:45	O/A (anti B 1:8)
$K4_{Cont}$	DCD	68 M, AB +ve	34:10	O/B (anti A 1:16)
$K5_{Cont}$	DCD	61 M, A –ve	23:40	A/O (Anti A 1:16; anti B 1:16)

DCD = donation after circulatory death; DBD = donation after brain death; M = male; F = Female; CIT = defined as the time from when the organ is perfused with cold preservation fluid within the donor at the time of retrieval to the time the organ commences ex-vivo normothermic perfusion; PRBC's = packed red blood cells. The above table shows donor type (DCD/DBD), age, sex, blood group, cold ischaemia time, and model tested with blood group of FFP with initial titre values for each experiment.

Table 2: **Demographic data of human kidneys for the HLAi, ABOi and Control (Cont) groups.**

dose of 600 ug of W6/32 anti-class 1 HLA. This dose of antibody was previously determined from the results of a complement dependent cytotoxicity test (See Supplementary S5). Fig. 3 shows that approximately 40 min after addition of W6/32 antibody, all three kidneys suffered a significant reduction in RBFi, characterised macroscopically by a dusky and mottled appearance in all three kidneys. Histologically, all kidneys demonstrated evidence of acute tubular injury (ranging from moderate to severe) with a varying degree of donor vasculopathy (mild to severe) with multifocal and linear complement glomerular capillary C4d deposition on immunofluorescence staining. Additionally, $K3_{HLAi}$ demonstrated evidence of microscopic thrombi in the peritubular capillaries. $K2_{HLAi}$ demonstrated interstitial oedema and glomerular capillary congestion possibly suggesting the presence of a thrombus. Upon opening the renal artery in both $K2_{HLAi}$ and $K3_{HLAi}$, visible clot was evident, and the kidneys appeared mottled. Fig. 4 demonstrates RBFi against perfusion time for $K1_{HLAi}$ and $K1_{Cont.}$ These were a pair of kidneys from the same donor, hence providing an ideal opportunity to randomly allocate a kidney to either an intervention or control experiment. $K1_{HLAi}$ suffered a rapid loss of renal blood flow index approximately 30 min after injecting W6/32 antibody into the circuit with low titre FFP. In contrast, its control $K1_{HLAi}$, maintained an improving blood flow with low titre FFP but no antibody added.

	Control group (n = 5)	ABOi group (n = 4)	HLAi group (n = 3)	*p* value
Donor age (years)	65 (53–68)	64 (59–69)	67 (47–67)	0.89[a]
Donor gender				
Male	4 (80%)	0 (0%)	2 (66.7%)	0.05[b]
Female	1 (20%)	4 (100%)	1 (33.3%)	
Donor type				
DCD	2 (40%)	1 (25%)	1 (33.3%)	0.89[b]
DBD	3 (60%)	3 (75%)	2 (66.7%)	
Blood group				
A	2 (40%)	3 (75%)	0 (0%)	0.13[b]
B	0 (0%)	1 (25%)	0 (0%)	
O	2 (40%)	0 (0%)	3 (100%)	
AB	1 (20%)	0 (0%)		
Cold ischaemia time (mins)	1845	1979	929	0.15[a]

The above table shows median donor age (IQR); gender, donor type: DCD = donation after circulatory death; DBD = donation after brain death, kidney blood group, cold ischaemia time = defined as the time from when the organ is perfused with cold preservation fluid within the donor at the time of retrieval to the time the organ commences ex-vivo normothermic perfusion. [a]Kruskal–Wallis test. [b]Chi-squared test.

Table 3: **Comparative demographic data of human kidneys for the HLAi, ABOi and control groups.**

ABOi group with high titre anti A and/or anti B antibodies

All kidneys in this group reached stabilisation with respect to RBFi prior to intervention with FFP containing a pre-determined antibody titre level, injected into the circuit. Allowing for circuit volume dilution effect the final representative antibody titres were as follows: $K1_{ABOi}$ 1:32–64 (anti A); $K2_{ABOi}$ 1:64–128 (anti A); $K3_{ABOi}$ 1:32–64 (anti A); $K4_{ABOi}$ 1:128–256 (anti B). Fig. 5 shows that both $K1_{ABOi}$ and $K3_{ABOI}$ had a gradual reduction in RBFi just over an hour after adding FFP. $K1_{ABOi}$ showed evidence of partial collapse with some recovery. This could be because the titres were too low (dilution effect) hence not adequate to cause thrombosis. The only other difference was that this kidney (Blood Group A) was inadvertently given group A packed cells (instead of O) which probably resulted in antibodies binding to red cell instead of binding to the kidney. Compared to these transient changes, $K2_{ABOi}$ and $K4_{ABOi}$ (which had the highest final circuit titres) demonstrated a marked reduction in RBFi within 45 min and 1 hr:20 min after addition of FFP, respectively. The reduction in RBFi of $K2_{ABOi}$ was markedly drastic. All kidneys in this group, macroscopically demonstrated a dusky, mottled appearance similar to $K2_{ABOi}$ shown in Fig. 5. Histologically, all kidneys showed evidence of moderate to severe acute tubular injury with donor vasculopathy. Although C4d immunostaining was negative for all these kidneys, $K2_{ABOi}$ demonstrated congested ischaemic glomeruli throughout, along with congested peritubular capillaries with evidence of microthrombi.

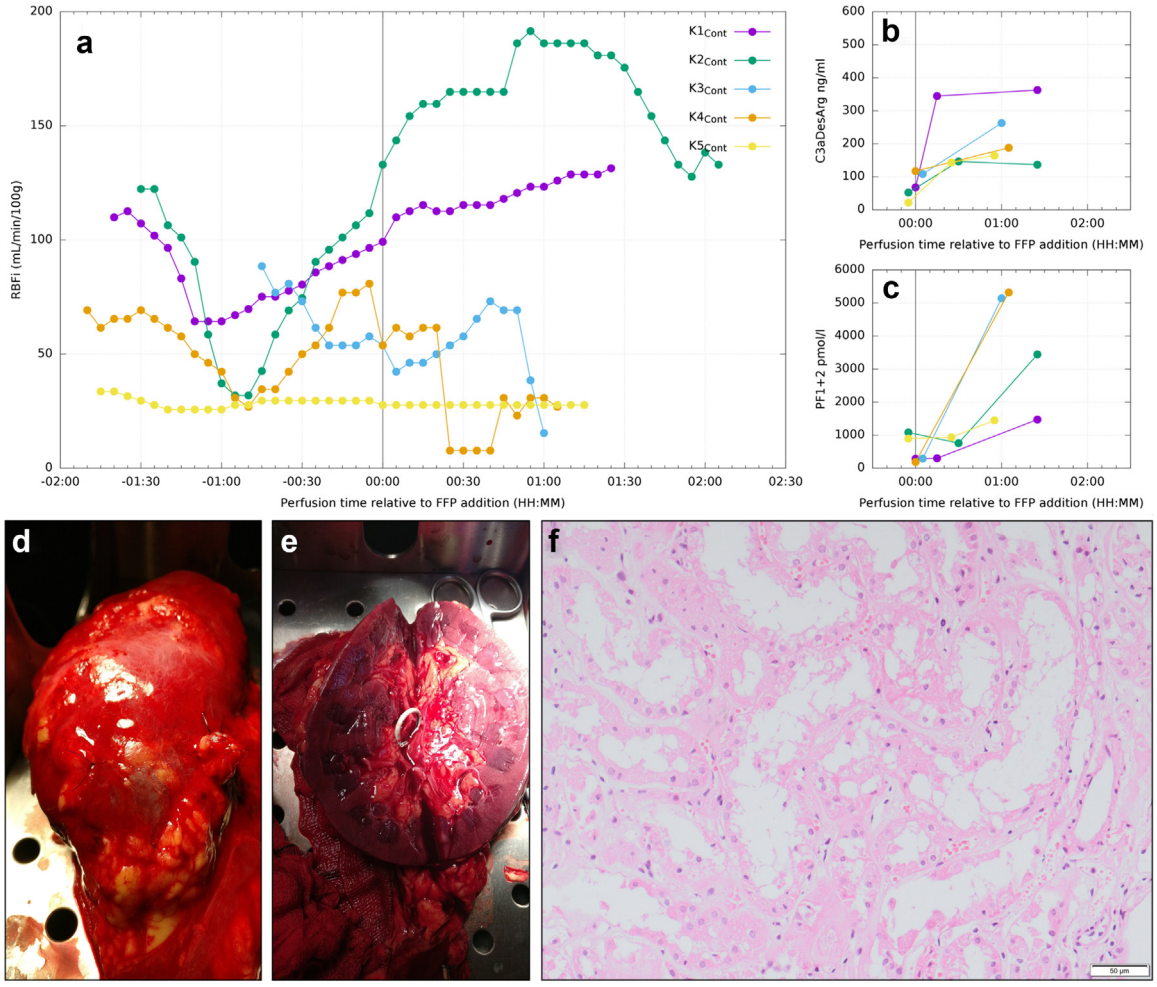

Fig. 2: **Control experiments: haemodyamic EVNP parameters, macroscopic appearance, and histology (H&E ×20).** (a) Renal blood flow index (mL/min/100 g) versus machine perfusion time (HH:MM) for control kidney experiments $K1_{Cont}$ – $K5_{Cont}$ with time 00:00 being the point of addition of (low titre <1:8) FFP. (b) and (c) C3a DesArg and PF 1 + 2 versus machine perfusion time (HH:MM) for $K1_{Cont}$ – $K5_{Cont}$ kidneys, respectively. Macroscopic appearance of $K1_{Cont}$: (d) post-addition of low titre FFP showing a pink well perfused kidney; (e) no visible thrombi present on cut section and (f) acute tubular injury on histology.

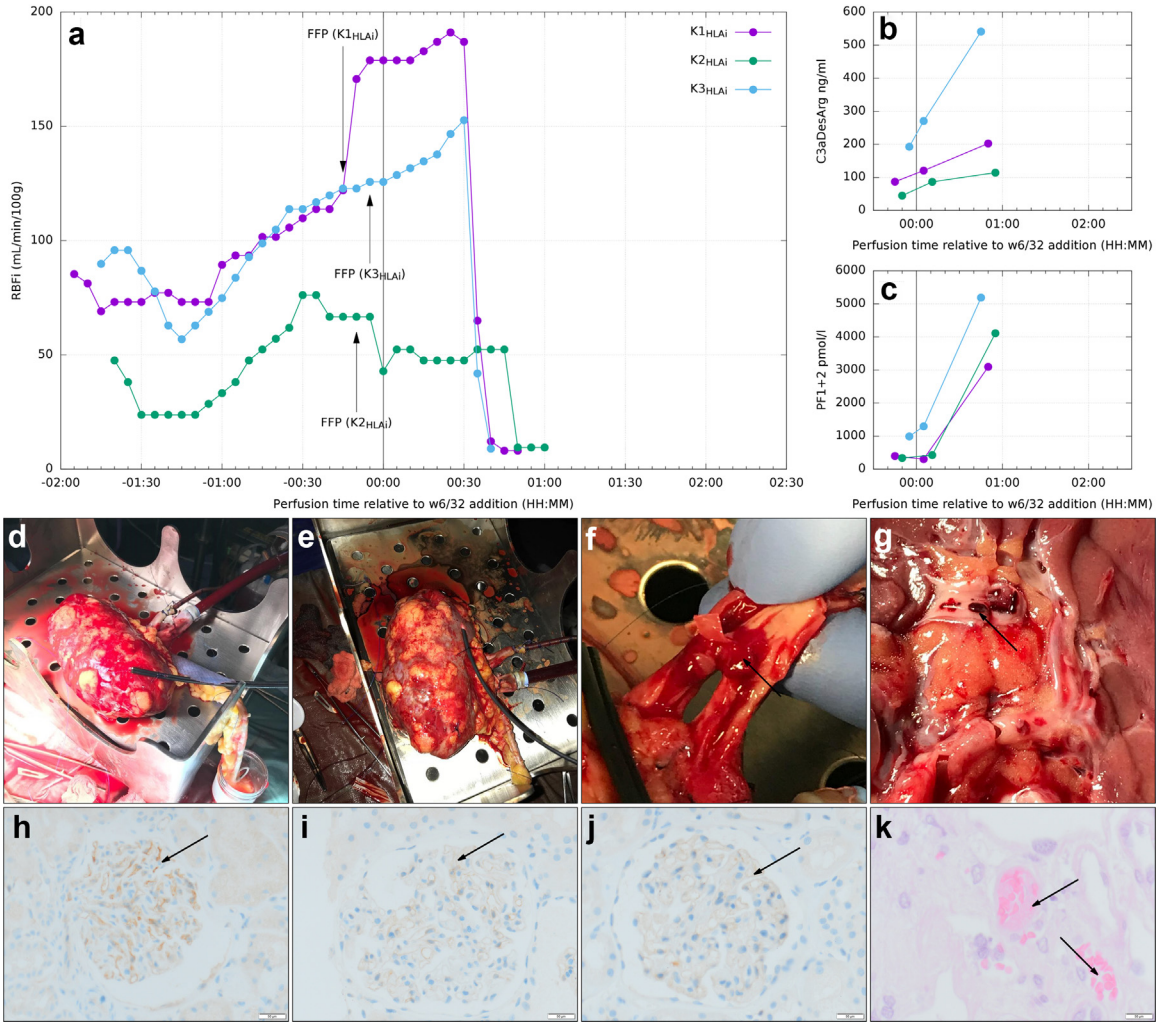

***Fig.* 3: HLAi AMR model: haemodyamic EVNP parameters, macroscopic appearance, and histology (C4d immunostaining and H&E staining ×20 magnification).** (a) Renal blood flow index (mL/min/100 g) versus machine perfusion time (HH:MM) for $K1_{HLAi}$, $K2_{HLAi}$ and $K3_{HLAi}$ with arrows indicating point of addition of FFP and time 00:00 indicating the point of addition of 600 ug of W6/32 anti-class 1 HLA antibody into the circuit. (b) and (c) C3a DesArg and PF 1 + 2 versus machine perfusion time (HH:MM) for $K1_{HLAi}$ – $K3_{HLAi}$, respectively. Macroscopic appearance $K3_{HLAi}$: (d) pre-addition of W6/32 antibody showing a pink, well perfused kidney; (e) post-addition of W6/32 antibody showing mottled, dusky kidney with appearance of fresh clot in the main renal artery (f) and within the renal parenchyma (g). Histology (h)–(j) demonstrated multifocal linear C4d glomerular capillary in $K1_{HLAi}$ – $K3_{HLAi}$ respectively with $K3_{HLAi}$ demonstrating presence of PTC microscopic thrombi (k).

All three groups demonstrated an increase in complement and coagulation activation. Although variables such as differing cold ischemia times between organs (hence the degree of ischaemia-reperfusion injury which also activate these processes), we could not demonstrate a difference in these metabolic responses according to experimental group.

Discussion

Antibody-mediated rejection (AMR) due to the presence of donor-specific antibodies against HLA or antibodies against blood group antigens can present an immunological barrier to transplantation and can cause significant allograft injury with graft loss.[35,36] Despite performing antibody-incompatible transplantation using established clinical protocols, antibody levels can return post transplantation causing early aggressive rejection and significant morbidity.[20,37] Currently there are no human models of AMR offering the potential to investigate alternative organ targeted approaches to treat AMR. Herein, using clinical grade bypass machine perfusion technology and human kidneys we report a model demonstrating a reproducible phenotype of

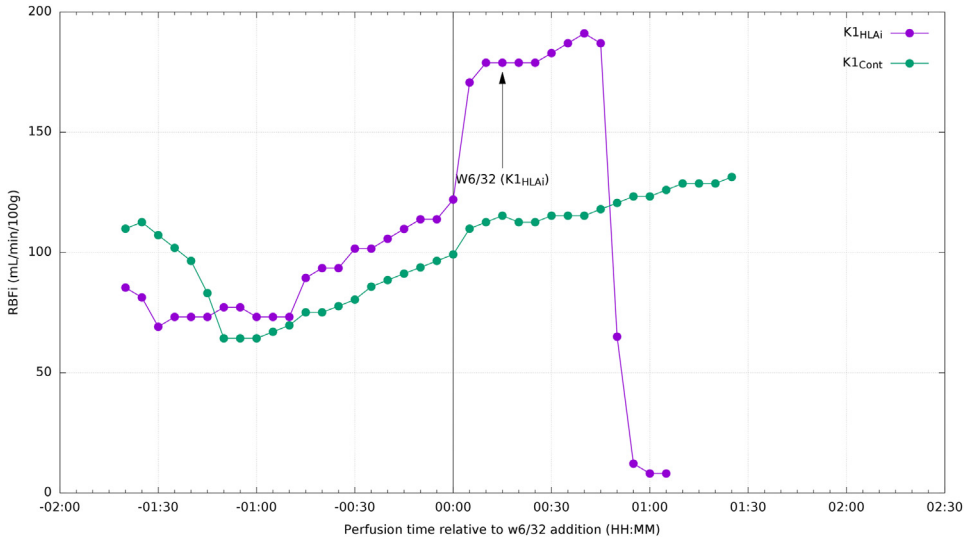

Fig. 4: **EVNP haemodynamic parameters for a pair of kidneys from the same donor ($K1_{HLAi}$ and $K1_{Cont}$).** Renal blood flow index (mL/min/100 g) versus machine perfusion time (HH:MM) for a pair of kidneys from the same donor $K1_{HLA}$i, $K1_{Cont}$ with time 00:00 indicating the point of addition of low titre FFP and arrow indicating addition of 600 ug of W6/32 anti-class 1 HLA antibody into the circuit for the experimental kidney K1HLAi (no antibody for $K1_{Cont}$).

antibody-mediated rejection using antibodies targeted against human HLA Class 1 (HLAi model) and blood group antigens A and B (ABOi model).

In the HLAi AMR model the consistent feature to indicate anti-class 1 HLA antibody binding to renal endothelium was the presence of immunofluorescent glomerular C4d deposition with haemodynamic blood flow collapse. One of these kidneys confirmed histological evidence of microvascular peritubular thrombosis and two kidneys showed evidence of macroscopic thrombus in the renal artery. The high titre ABOi AMR model experiment also demonstrated a marked reduction in renal blood flow approximately 40 min after FFP was added and showed evidence of histological microvascular thrombi in the peritubular capillaries but with C4d negative staining Although two controls did demonstrate a gradual decline in RBFi at end of the experimental phase, consistent with previous observations of warm kidney perfusion,[38] the phenotype associated with the HLAi and ABOi models were different and very distinct. All controls were also C4d negative with no evidence of thrombosis.

All three groups demonstrated activation of complement and coagulation but with wide variations in C3a desArg levels and PT fragments 1 + 2 between the kidneys.

With respect to the experimental AMR phenotype in the two models, there are features present that would suggest AMR, namely, thrombosis, acute tubular injury and haemodynamic collapse (with C4d glomerular deposition in the HLA kidney group). Although glomerular C4d staining is being increasingly accepted as a feature of AMR,[39,40] the current Banff criteria[41] does not apply to our reductionist model devoid of leucocytes, platelets and macrophages. C4d has been widely used as evidence of antibody interaction with the vascular endothelium since the early 1990's.[42,43] However, studies suggest that C4d is a specific but insensitive marker of AMR.[7,44] Additionally, the interpretation of C4d positivity is also time-dependent post-transplantation as studies have reported biopsy proven C4d positivity occurring within 1 h of re-perfusion in ABOi transplants without any immediate evidence of rejection that may not manifest even one month later.[45] In the control group there is evidence of complement activation due to the presence of C3a desArg in the circuit independent of the presence of W6/32 antibody or high titre anti-A/anti-B antibody. This is likely to reflect alternate pathway activation related to the experimental circuit. However, for the HLAi kidney group, C4d provides a better discriminator of the presence of anti-HLA antibody than C3a desArg. The presence of C4d in this group may suggest a possible upstream mediated complement dependent activation of the coagulation pathway that eventually led to a thrombotic microangiopathy (TMA). Studies have reported a glomerular distribution of C4d deposition in TMA compared to the typical characteristic peritubular C4d deposition seen in AMR. They also report co-localisation of C4d with C5b-9.[46,47] The MAC complex (C5b-9) is known to lead to increase tissue factor expression on endothelial cells in vitro, thus activating coagulation and promoting thrombin generation,[48] the relevance of this, in vivo however, is unproven. This suggests the complex interplay that occurs when antibody

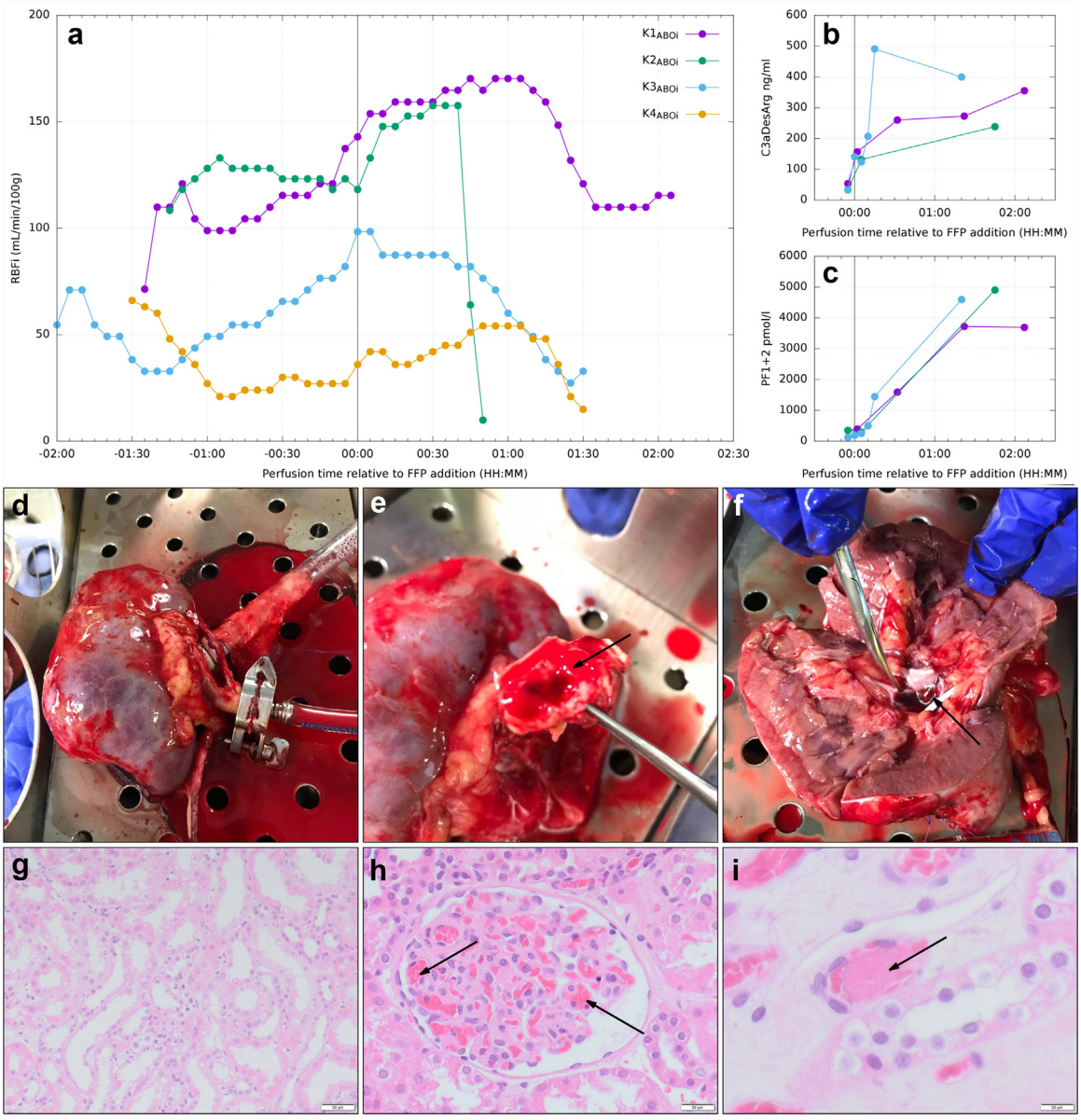

Fig. 5: **ABOi AMR model: haemodyamic EVNP parameters, macroscopic appearance, and histology (H&E staining ×20 magnification).** (a) Renal blood flow index (mL/min/100 g) versus machine perfusion time (HH:MM) for $K1_{ABOi}$ – $K4_{ABOi}$, with time 00:00 being the point of addition of high-titre FFP to circuit; (b) and (c) C3a DesArg and PF 1 + 2 versus machine perfusion time (HH:MM) for $K1_{ABOi}$ – $K3_{ABOi}$, respectively (note $K4_{ABOi}$ data was obtained but misplaced); (d) Macroscopic appearance of $K2_{ABOi}$ following catastrophic reduction in RBFi showing a dusky mottled kidney; with evidence of fresh clot in the main renal artery (e); and within the renal vein (f). Histology of $K2_{ABOi}$ showing: (g) acute tubular injury; (h) congested glomerular capillaries with red blood cells; (i) peritubular capillary microscopic thrombi.

binds to endothelium activating the coagulation cascade via complement. Our findings potentially help to further support this hypothesis. In the high titre ABO kidney, there was no C4d positivity despite a drastic reduction in blood flow and presence of peritubular thrombus implying a different pathway in the setting of blood group incompatibility. The importance of C4d positivity in ABOi renal recipients has been previously reported with no evidence of graft dysfunction.[49] Therefore, the presence and role of C4d positivity in HLAi AMR may have different mechanistic implications to that in ABOi AMR where C4d was negative. The amplitude of complement activation in the context of antibody binding is dependent on several factors including, the type and amount of

antibody isotype (IgM, IgG3 and IgG1 are more potent inducers of the classical pathway), the intensity of the antibody-antigen binding and the presence and concentration of complement regulatory proteins such as DAF, CD 59.[50] Our method for establishing blood group antibody titres detects both IgG and IgM. IgM is the more predominant subclass blood group antibody compared to IgG[51] and has been shown to activate the alternate complement pathway.[52] This could explain the lack of C4d deposition in ABOi kidney experiments. In this regard, determining C3d deposition in C4d negative cases, could indicate whether the alternative or lectin pathways were activated.

Complement and coagulation activation was demonstrated in the two experimental groups in addition to the control group, hence these parameters could not be used a discriminator for AMR. Several factors could account for this. The variation in C3a desArg and PT fragments 1 + 2 could be due to the kidneys having varied cold ischaemia times and donor characteristics, such as amount of ABO antigen expression, demonstrating different ischaemia-reperfusion responses whilst undergoing warm perfusion. In addition, ex vivo bypass circuitry perfusate flows may also be a contributing factor as cardiopulmonary bypass has been widely known to activate alternate complement pathways in patients undergoing cardiac surgery.[53,54] In our model, the perfusion tubing and machine-related factors were the same for each kidney so any circuit contribution to complement activation was similar for all experiments. Different kidneys may be synthesising different levels of local C3 which can also contribute to the overall intensity of complement activation[55] but the relative contributions of local versus FFP derived complement activation would be difficult to ascertain in our model. Another explanation for the variations in these levels could be that the kidneys are expressing different levels of membrane bound complement regulatory proteins on the endothelium (e.g. CD46, CD59, DAF) and thus offering different levels of protection against any antibody effect or ischaemia reperfusion injury. In addition, renal derived soluble complement regulators are also produced, including Factor H (which accelerates the decay of C3 convertase) and C4b binding protein (which accelerates the decay of C3 and C5 convertase) and hence these may confer additional protective benefit to membrane bound complement regulators.

Although there have been reports of animal AMR models, they have limitations for human translation as they may not necessarily embody the full spectrum of donor demographics and organ physiology associated with deceased human organ transplantation. Additionally, certain animal strains used in AMR models may have different complement levels and regulatory proteins compared to human organs making clinical translation from these models challenging.[56,57]

Our model offers clinical value by providing a platform for the manipulation and testing of novel organ-specific strategies targeting the renal endothelium to help prevent the deleterious effects of AMR. Strategies include using locally acting cytoprotective agents that have previously been shown in animal studies of AMR to inhibit complement and coagulation activation.[26,58] In addition, regenerative cellular therapy could be used to evaluate the ability of the kidney to repair and regenerate. Such therapies have shown beneficial effect in warm perfusion kidney models of ischaemia-reperfusion injury[59–61] but have not been proven in the context of AMR. Finally, testing of gene therapy and selective silencing of endothelial HLA expression may offer an alternative approach to protecting the organ against AMR.[62–67]

Refinements in our model would be necessary to test such strategies including the infusion of therapeutic and regenerative agents into the kidney to allow binding to the endothelium prior to the commencement of warm machine perfusion. The integration and subsequent protective function of these perfusion therapies are metabolically active processes that may require prolonged warm perfusion times (>24 h). Additionally, upregulation of protective membrane bound complement regulatory proteins (e.g.CD59, CD55/DAF, CD46), gene transcription and protein translation are further time-dependent processes that may also occur during the testing of these strategies.[68–70] By attempting to protect the renal endothelium prior to transplantation, the immune response to antibody effect may be ameliorated, inducing artificial accommodation of the graft that may allow transplantation across difficult immunological barriers.

There are limitations to our study including the use of a wide range of declined human kidneys for research purposes with variable donor characteristics, quality, and cold ischaemia times (which ranged from approximately 11 h–54 h). Inevitably this also relies on the availability of suitably retrieved organs amenable to EVNP. To mitigate against these limitations, we aimed to standardise minimum baseline haemodynamic parameters during warm perfusion prior to our intervention.

The presence of leucocytes in whole blood may contribute to the ischaemia reperfusion injury masking any experimental effect hence our choice to use packed red cells as the blood based perfusate solution devoid of leucocytes (which is also used in the clinical EVNP setting). Interestingly, kidneys have been shown to have donor-derived passenger leucocytes. Other experiments performed at our institution have shown (Phillips B, institution, personal), that the perfusate contains many donor-derived leucocytes which may contribute to an element of ischaemia-reperfusion injury in these declined kidneys with an already prolonged cold ischaemia time thus further adding to the complement activation, however this needs further characterisation.

Hence in trying to further reduce the effects of any ischaemia-reperfusion injury on our experimental conditions it was critical for us to have a period of stable renal blood flow in the kidney during EVNP *prior* to adding the FFP (if the kidney did not reach our criteria for RBFi stability it would have been discarded for experimental intervention). Hence, we endeavoured to minimise the influence of any EVNP-associated ischaemia reperfusion injury on the AMR induced experimental conditions.

The W6/32 monoclonal antibody reacts with all human HLA class 1 (HLA-A, -B, and -C).[71] Although a murine antibody, it has three major advantages over other sources of HLA antibody. Firstly, it is available as a highly purified product, making it ideal for reproducible introduction into the perfusion circuit; secondly, as it binds to all anti-human class I molecules this enabled us to use all kidneys, not just those of a particular HLA type; thirdly, red blood cells do not express Class 1 so the antibody will not cause red cell lysis within the perfusion circuit. Although using a "pan" anti-class 1 antibody maybe useful in an experimental model of severe antibody-mediated rejection, this may not necessarily represent the entire clinical scenario where an anti-class 1 antibody may not have the ability to bind with all HLA class 1. Additionally, we did not test anti HLA Class II antibody in our AMR model which has been reported to be the predominant de novo donor specific antibody produced after transplantation in unsensitised renal recipients.[72–74]

The thawing, refreezing, and storage of clinically discarded FFP at –70 to –80 °C may also affect complement and coagulation protein stability and physiological activity. However, we demonstrated that W6/32 antibody causes lysis of leucocytes in a clinically validated CDC crossmatch test when used at the concentration it is circulating in the EVNP circuit, but only when FFP (also at the dilution used in the circuit) was present, confirming that both reagents are appropriate in this experimental setting, and most importantly that the complement in these FFP bags is physiologically active. Further studies have also shown stability of these proteins following thawing and long-term storage once frozen.[75,76] Finally, our small sample size, and the inability to perform multivariate analyses, is also a limitation that prevented us from risk-adjusting for confounding factors. However, we consider it unlikely that donor age and sex, deceased donor type (DCD/DBD) and cold ischaemia times acted as confound factors, since these were equally represented in each group. Despite these limitations, our data provides an encouraging foundation for larger studies.

In summary, we have developed a clinically relevant human model of antibody-mediated rejection that is a prerequisite to establishing any platform for organ-specific machine perfusion therapeutic and pre-conditioning paradigms to protect against the deleterious effects of antibody against the transplanted graft. This may help in reducing the burden of current systemic treatment modalities.

Contributors

P.C. conducted and helped devise all experiments, collected data, and wrote the manuscript as primary author. B.P., R.U., D.B., N.K. conducted EVNP experiments and collected data and helped edit manuscript; T.M., L.W., D.V. helped supply FFP and red blood packed cells and L.W., D.V., and O.S. helped perform FFP and antibody titre experiments. R.P. processed and analysed the biopsies. K.P. and B.H. processed and analysed the complement and coagulation data; C.C. helped supervise and set up EVNP; A.D., and N.M. as senior authors helped devise methodology and edited the final manuscript. Underlying data was verified by P.C., B.P., D.B., O.S., R.P., K.P., B.H., C.C., A.D., N.M. All authors have read and approved the final version of the manuscript.

Data sharing statement

All experimental data collected during the study will be made available on request to pankaj.chandak@kcl.ac.uk.

Declaration of interests

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2022.104365.

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