

RESEARCH ARTICLE

Human Alpha-1-Antitrypsin (hAAT) therapy reduces renal dysfunction and acute tubular necrosis in a murine model of bilateral kidney ischemia-reperfusion injury

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Abstract

Several lines of evidence have demonstrated the anti-inflammatory and cytoprotective effects of alpha-1-antitrypsin (AAT), the major serum serine protease inhibitor. The aim of the present study was to investigate the effects of human AAT (hAAT) monotherapy during the early and recovery phase of ischemia-induced acute kidney injury. Mild renal ischemia-reperfusion (I/R) injury was induced in male C57Bl/6 mice by bilateral clamping of the renal artery and vein for 20 min. hAAT (80 mg/kg, Prolastin[®]) was administered daily intraperitoneally (i.p.) from day -1 until day 7 after surgery. Control animals received the same amount of human serum albumin (hAlb). Plasma, urine and kidneys were collected at 2h, 1, 2, 3, 8 and 15 days after reperfusion for histological and biochemical analysis. hAAT partially preserved renal function and tubular integrity after induction of bilateral kidney I/R injury, which was accompanied with reduced renal influx of macrophages and a significant decrease of neutrophil gelatinase-associated lipocalin (NGAL) protein levels in urine and plasma. During the recovery phase, hAAT significantly decreased kidney injury molecule-1 (KIM-1) protein levels in urine but showed no significant effect on renal fibrosis. Although the observed effect size of hAAT administration was limited and therefore the clinical relevance of our findings should be evaluated carefully, these data support the potential of this natural protein to ameliorate ischemic and inflammatory conditions.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Renal ischemia-reperfusion (I/R) injury is a frequent cause of acute kidney injury (AKI) in a variety of clinical conditions such as cardiac or aortic surgery and kidney transplantation, and is associated with significant morbidity and mortality. Ischemic injury of the kidney causes the release of damage-associated molecular patterns (DAMPs) by impaired endothelial and tubular epithelial cells [1]. These DAMPs are rapidly sensed by pattern recognition receptors, which together with the formation of reactive oxygen species after reperfusion lead to an inflammatory response. Therefore, the focus of current research is to control innate immune pathways in order to reduce I/R damage.

Alpha-1-antitrypsin (AAT) is the most abundant serum serine protease inhibitor, primarily produced by the liver. Its main physiological role is to inhibit the activity of different endogenous serine proteases, such as neutrophil-derived elastase and proteinase-3 [2–4]. These proteolytic enzymes contribute to the inflammatory response by activating pro-cytokines and through the formation of DAMPs. Due to its anti-protease activity, AAT can inhibit these pathways, consequently exerting anti-inflammatory and tissue-protective effects. During acute-phase responses, such as injury or infection, circulating AAT levels can increase approximately four-fold. In addition, AAT levels are up-regulated during hypoxia [5] and are known to be increased in plasma from patients with acute myocardial infarction [6, 7] and in urine of patients with AKI [8], probably as an endogenous protective response against ischemic injury. Accordingly, human AAT (hAAT) given to mice during acute myocardial I/R injury limited the infarct size and protected from adverse cardiac remodeling [9]. Moreover, there is a growing body of evidence supporting the anti-inflammatory and cytoprotective effects of this acute-phase reactant in a wide range of *in vitro* and *in vivo* experimental models [10]. The cellular targets of AAT mostly include cells of the innate immune system, such as neutrophils and macrophages, as well as B lymphocytes and dendritic cells which are involved in the adaptive immune response. However, its mechanism of action is not completely understood, and some studies suggest that the protective effects of AAT are independent of its serine protease inhibiting activity [11].

Given the pivotal role of the early inflammatory response in the pathogenesis of ischemic injury, we sought to investigate the effects of hAAT monotherapy on both AKI and the kidney repair process after ischemic insult. To address these issues we performed a mouse model of bilateral kidney I/R injury.

Materials and methods

Animals

All animal procedures were approved by the Animal Ethics Committee of the Radboud university (Nijmegen, the Netherlands; RU-DEC 2011–049 / 2013–198). Handling of animals was performed according to the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive (86/609/EEC). Male C57Bl/6N mice (Charles River, Sulzfeld, Germany) were housed at the Central Animal Facility of the Radboud University under specific pathogen-free conditions with *ad libitum* food and water.

Experimental bilateral kidney I/R model

All surgical procedures were performed on 8/9-week-old mice (22–28 g) using standard aseptic surgical techniques, with all efforts to minimize suffering. Carprofen [5 mg/kg body weight (b.w.)] was selected as a non-steroidal analgesic in all experimental groups and administered subcutaneously (s.c.) 30 min before the surgery, 24h and 48h after surgery. Anesthesia was

induced with 5% isoflurane in O₂/N₂O and subsequently kept at 2.5–3% during the operation. Mice were laparotomized and body temperature was maintained at 36.5–37°C. The renal vein and artery of both kidneys were freed from surrounding white adipose tissue and clamped with microvascular clamps (B-1V from S&T, Neuhausen, Switzerland) for 20 min. Absence of renal blood flow during clamping and subsequent renal reperfusion after releasing the clamp, was visually monitored by respectively the discoloring and re-coloring of the kidney. Animals that did not display a homogeneous and marked kidney color change or with high temperature ($\geq 38^\circ\text{C}$) during the surgical procedure were excluded from the study. In a pilot study to determine the appropriate ischemic time for this model within our experimental conditions, a sham-operation group ($n = 3$ animals) was included. Same surgical procedure, without clamping of the renal vessels, was performed on these animals. Sham-operated mice overcame the surgery without neither signs of sickness nor renal changes when compared to naïve animals: levels of plasma creatinine ($<12 \mu\text{mol/L}$ vs naïve animals $<12 \mu\text{mol/L}$), urine KIM-1 levels ($429.0 \pm 190.9 \text{ pg/mL}$ vs naïve animals $406.1 \pm 136.7 \text{ pg/mL}$; $P > 0.05$) and plasma NGAL levels ($241.3 \pm 77.9 \text{ ng/mL}$ vs naïve animals $161.3 \pm 41.1 \text{ ng/mL}$; $P > 0.05$).

Study design and treatment

Mice were randomly assigned to the experimental groups ($n = 6$ –8 animals per control groups and $n = 6$ –8 animals per hAAT groups) and placed in metabolic cages around 1 week before surgery (day 7 pre-op), immediately after the surgery, and at day 1, 2, 7, or 14 after reperfusion (post-op) to collect urine. Blood samples were obtained and mice were sacrificed by cervical dislocation at 2h and 1, 2, 3, 8, and 15 days after surgery.

Clinical grade human AAT (hAAT, Prolastin[®], Bayer Corporation) was dissolved in sterile water and administered intraperitoneally (i.p.) at a dose of 80 mg/kg (2 mg/mouse/day; injection volume of 200 μL) starting at day -1 (24h before the surgery), day 0 (30 min before the surgery) and then daily for a maximum of 7 days. Control animals received the same amount of human serum albumin (hAlb; injection volume of 200 μL) (Sigma-Aldrich) as control for human protein administration. Weight and well-being of the mice were monitored daily.

Tissue and blood handling

Blood samples were collected in heparin tubes and centrifuged at 1200 $\times g$ for 10 min at 10°C to obtain plasma. Protease inhibitors were added to urine samples after centrifugation at 3000 $\times g$ for 15 min at 4°C. Plasma and urine samples were stored at -20°C and -80°C, respectively. Kidneys were harvested and fixed in 4% paraformaldehyde in order to perform the histological analysis or snap frozen in liquid nitrogen to perform the immunohistochemical analysis and RNA isolation.

Renal function assessment

The renal function was determined by measuring creatinine and urea levels in plasma samples using routine standard clinical chemical methods by our hospital diagnostic facility.

Histological analysis

Fixed renal tissue was dehydrated and subsequently embedded in paraffin. Kidneys were cut at different latitudes into 4- μm sections, mounted on 3-aminopropyltriethoxysilane (APES)-coated slides and dried at 37°C for at least 24h. Sections were stained with periodic acid-Schiff (PAS). All the histopathological scores were made in 3 different areas: outer cortex (OC), cortico-medullary junction (CMJ) and inner medulla (IM). The percentage of damaged tubules in

these 3 areas was estimated as described previously [12] and according to the following criteria: cast formation, debris deposition in the tubular lumen and loss of the brush border from the proximal tubules in 10 randomly chosen, non-overlapping fields ($\times 400$ magnification). Lesions were graded on a scale from 0 to 5: 0 = normal, no proximal tubular damage; 1 = mild, involvement of less than 10% of the area; 2 = moderate, involvement of 10–25% of the area; 3 = severe, involvement of 25–50% of the area; 4 = very severe, involvement of 50–75% of the area; 5 = extensive damage, involvement of more than 75% of the area. The score was performed on blinded sections by two observers.

Kidney fibrosis

Tissue sections (4 μm) were deparaffinized, rehydrated and stained with 0.2% Picro-Sirius Red for 60 min for assessment of fibrotic tissue formation. Collagen fibers were coloured in bright red on a pale yellow background. Quantification of collagen-stained area (μm^2) was performed in non-overlapping fields ($\times 200$ magnification) throughout 2 different regions of the kidney (outer cortex and cortico-medullary junction) using the image software KS 400, and subsequently averaged. Analysis was performed on blinded sections by an observer.

Measurement of AKI markers in urine and plasma

Kidney injury molecule-1/T-cell Ig mucin protein-1 (KIM-1/TIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) protein levels were measured in urine and plasma using high sensitivity enzyme-linked immunosorbent assay (ELISA) kits (from R&D Systems Inc., Minneapolis, MN, USA).

Measurement of plasma hAAT levels and mouse anti-hAAT antibodies

Determination of plasma hAAT levels was performed using an ELISA kit (GenWay Biotech, Inc., San Diego, CA, USA). Detection of mouse anti-hAAT antibody levels in plasma was performed by ELISA as previously described [13].

Determination of inflammatory cells by Immunofluorescence (IF)

Frozen kidney sections (2 μm) were analyzed by IF as described previously [14]. Double stainings with a monoclonal antibody against the core protein of agrin (hamster anti-mouse; MI91) [15] (dilution 1:800) and against granulocytes (rat anti-mouse; Gr-1, Ly6G/LY-6C-FITC labeled) (BioLegend, San Diego, CA, USA) (dilution 1:100) or macrophages (rat anti-mouse; CD68) (AbD Serotec, Bio-Rad laboratories, Inc., Hercules, CA, USA) (dilution 1:1600) were performed. CD68 and MI91 were stained with goat anti-rat Alexa Fluor[®] 488-conjugated secondary antibody (Invitrogen, Paisley, UK) (dilution 1:200) and goat anti-hamster Cy[™]3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, PA, USA) (dilution 1:600), respectively. Granulocytes were counted in the kidney cortex sections in non-overlapping fields ($\times 400$ magnification) and macrophage staining was scored semi-quantitatively on a scale from 0 to 5 based on the extent of CD68 immunofluorescence staining by two observers on blinded sections.

RNA isolation from kidney tissue and real-time quantitative RT-qPCR

RNA was isolated from kidney tissue using the RNeasy Mini kit (Qiagen) and reverse transcribed (Transcription Kit; Roche Diagnostics, Mannheim, Germany). Interleukin-1 β (IL-1 β), macrophage galactose-type C-type lectin-1 (MGL-1), macrophage inflammatory protein-1 α (MIP-1 α), monocyte chemoattractant protein-1 (MCP-1), interferon regulatory factor 5

(IRF5), chemokine (C-X-C motif) receptor 2 (CXCR2), mouse keratinocyte-derived cytokine (KC), transforming growth factor- β (TGF- β), collagen-1 α (col-1 α), collagen-4 (col-4) and matrix metalloproteinase-9 (MMP-9) gene expression was determined by real-time quantitative PCR using SYBR Green SuperMix (Roche Diagnostics) on a MyiQ real-time PCR detection system (Bio-Rad Laboratories), as described previously [16]. Primer sequences for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the genes of interest can be found in [S1 Table](#). For each sample, differences in threshold cycle (Δ Ct) values were calculated by correcting the Ct of the gene of interest to the Ct of the housekeeping gene GAPDH. Results were expressed as Rq (fold change) = $2^{-\Delta\text{Ct (hAAT group)}} / \text{mean } 2^{-\Delta\text{Ct (control group)}}$.

Statistical analysis

Values are represented as box and whisker plots with median, 25th and 75th percentiles, and minimum and maximum values, or as mean \pm SEM. Data were analyzed using GraphPad Prism[®] (version 5.03 for Windows; Graphpad Software Inc., San Diego, CA). Spearman's correlation coefficient was used to quantify the relationship between two variables. Differences between experimental groups and changes of parameters in time were tested using the two-tailed Mann-Whitney U test when comparing two treatment groups at one time-point or two-way analysis of variance (ANOVA) of data after logarithmic normalization (factors time and treatment) followed by *Bonferroni* multiple comparison post-test, as appropriate (see Figure legends). *P* values less than 0.05 were considered significant.

Results

hAAT protects against I/R-induced renal dysfunction

I/R injury significantly impaired kidney function, as reflected by a marked increase in plasma creatinine and urea levels. The observed increase of these parameters peaked at days 2 and 3 after reperfusion, followed by a return to basal levels with almost complete recovery at day 15 post-op. Treatment with hAAT ameliorated acute renal dysfunction, partly protecting against renal failure as indicated by significantly lower levels of plasma urea at day 2 after reperfusion ([Fig 1A](#)) and a tendency to lower plasma creatinine levels compared to the control group ([Fig 1B](#)).

hAAT decreases I/R-induced tubular damage

Two days after inducing I/R injury, kidneys showed widespread tubular damage over the cortex, cortico-medullary junction and medullary region, reflected by a significant increase in the renal damage score ([Fig 2G](#)) when compared to naïve animals ([Fig 2](#), panels A and D). These damaged and necrotic tubules showed either cast formation, debris deposition in the tubular lumen, or shedding of the brush border from the proximal tubules ([Fig 2B](#)). One week after the ischemic insult, tubules started to recover as can be concluded from the repaired brush border membranes that were observed during the late stages of kidney I/R damage ([Fig 2C](#)). Kidneys from hAAT-treated mice displayed less extensive tubular necrosis compared to the hAlb-treated control animals ([Fig 2](#), panels E and F), with a significantly reduced histological damage score in the cortico-medullary junction and inner medulla at day 2 after kidney I/R injury ([Fig 2G](#)).

hAAT ameliorates Acute Kidney Injury (AKI)

To assess AKI, we measured the concentration of the acute renal injury markers KIM-1 and NGAL in urine and plasma. When compared to the basal levels at day 7 pre-op, 20-min renal

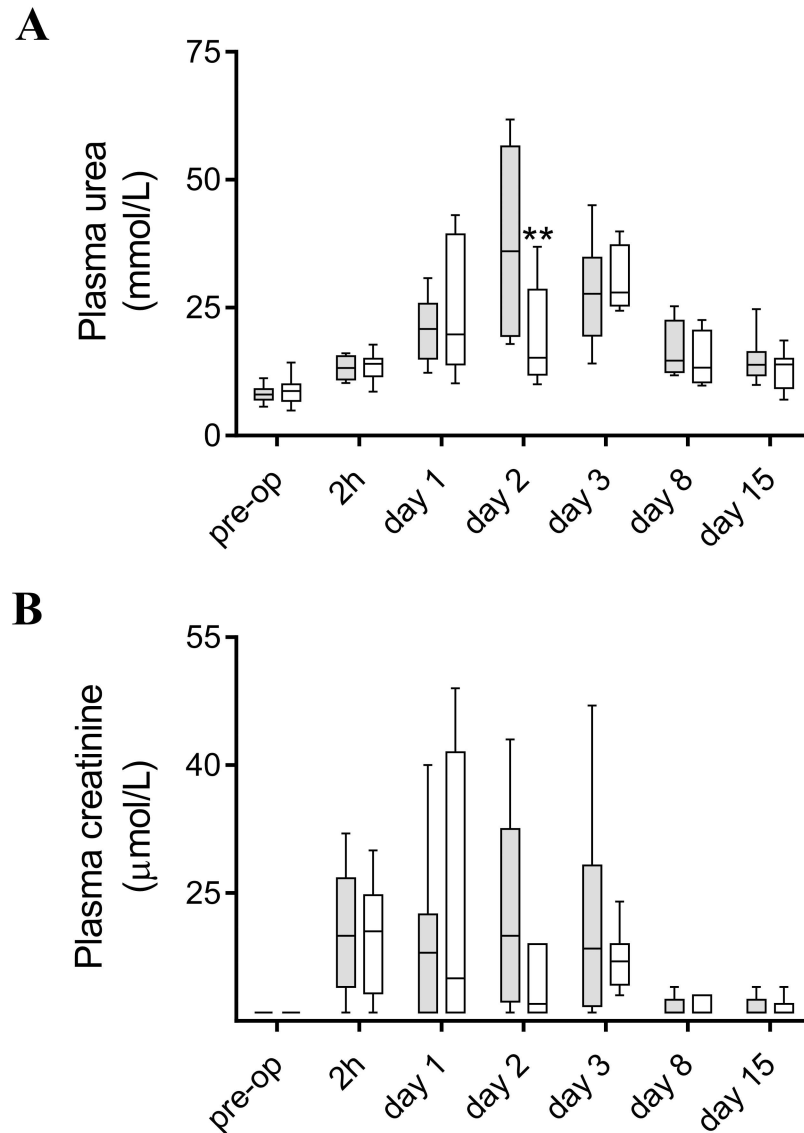


Fig 1. hAAT (80 mg/kg/day; i.p.) treatment improves renal function after I/R injury (IRI). **A.** Plasma urea levels at day 7 pre-op and at different time-points after IRI. **B.** Plasma creatinine levels at day 7 pre-op and at different time-points after IRI. Grey boxes represent the control group treated with hAlb. White boxes represent the group treated with hAAT. Two-way analysis of variance (ANOVA) followed by *Bonferroni* post-test. ** $P < 0.01$ ($n = 6-8$ animals per group).

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ischemia significantly increased the protein levels of both AKI markers KIM-1 and NGAL in urine and plasma (Fig 3). Maximal expression of these proteins was observed during the early phase of the renal I/R injury.

Treatment with hAAT ameliorated AKI, as indicated by a smaller rise in NGAL protein levels in urine and plasma in the early phase of the kidney I/R damage (Fig 3, panels A and B). In addition, hAAT therapy significantly reduced the urine protein levels of KIM-1 at day 15, during the recovery phase of the renal ischemic damage (Fig 3C).

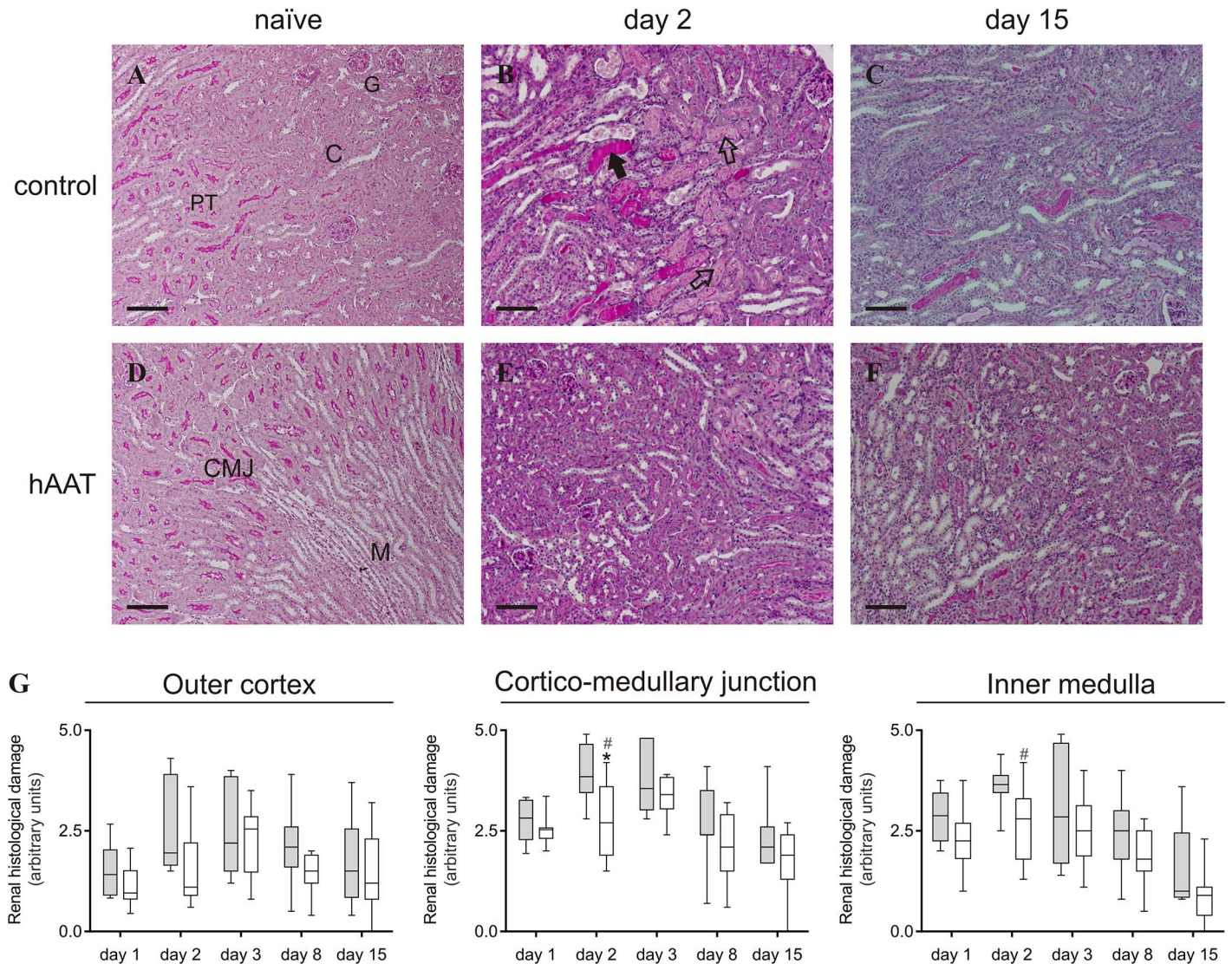


Fig 2. hAAT (80 mg/kg/day; i.p.) treatment decreases acute tubular necrosis after I/R Injury (IRI). **A-F.** PAS-stained frontal sections of mouse kidney: **A, D** PAS-stained frontal sections of naïve mouse kidney without any histological damage and with the intact brush border in the proximal tubules. **B, C** representative images of ischemic kidney of control mouse at different time-points after IRI. **E, F** representative images of ischemic kidney of mouse treated with hAAT at different time-points after IRI. C = cortex; CMJ = cortico-medullary junction; M = medullary region; PT = proximal tubules; G = glomeruli. The solid arrow indicates areas of cast formation. Open arrows indicate debris deposition in the tubular lumen. Scale bar = 100 μ m (original magnification $\times 100$). **G.** Kidney histological damage score. The percentage of damaged tubules in 3 different areas of the kidney was estimated using a 5-point scale. Grey boxes represent the control group treated with hAlb. White boxes represent the group treated with hAAT. Two-way analysis of variance (ANOVA) followed by *Bonferroni* post-test. * $P < 0.05$. Mann-Whitney U test (two-tailed) to analyze the effect of treatment at each time-point. # $P < 0.05$ ($n = 6-8$ animals per group).

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Effects of hAAT on granulocyte and macrophage infiltration in post-ischemic kidneys

In order to evaluate the effect of hAAT treatment on leukocyte influx, we evaluated the Gr-1⁺ and CD68⁺ cell staining in post-ischemic kidneys during the early stage of the kidney I/R damage. As depicted in Fig 4, mice subjected to 20-min renal ischemia displayed a significant increase in granulocyte and macrophage infiltration, which peaked at day 1 and 3 after

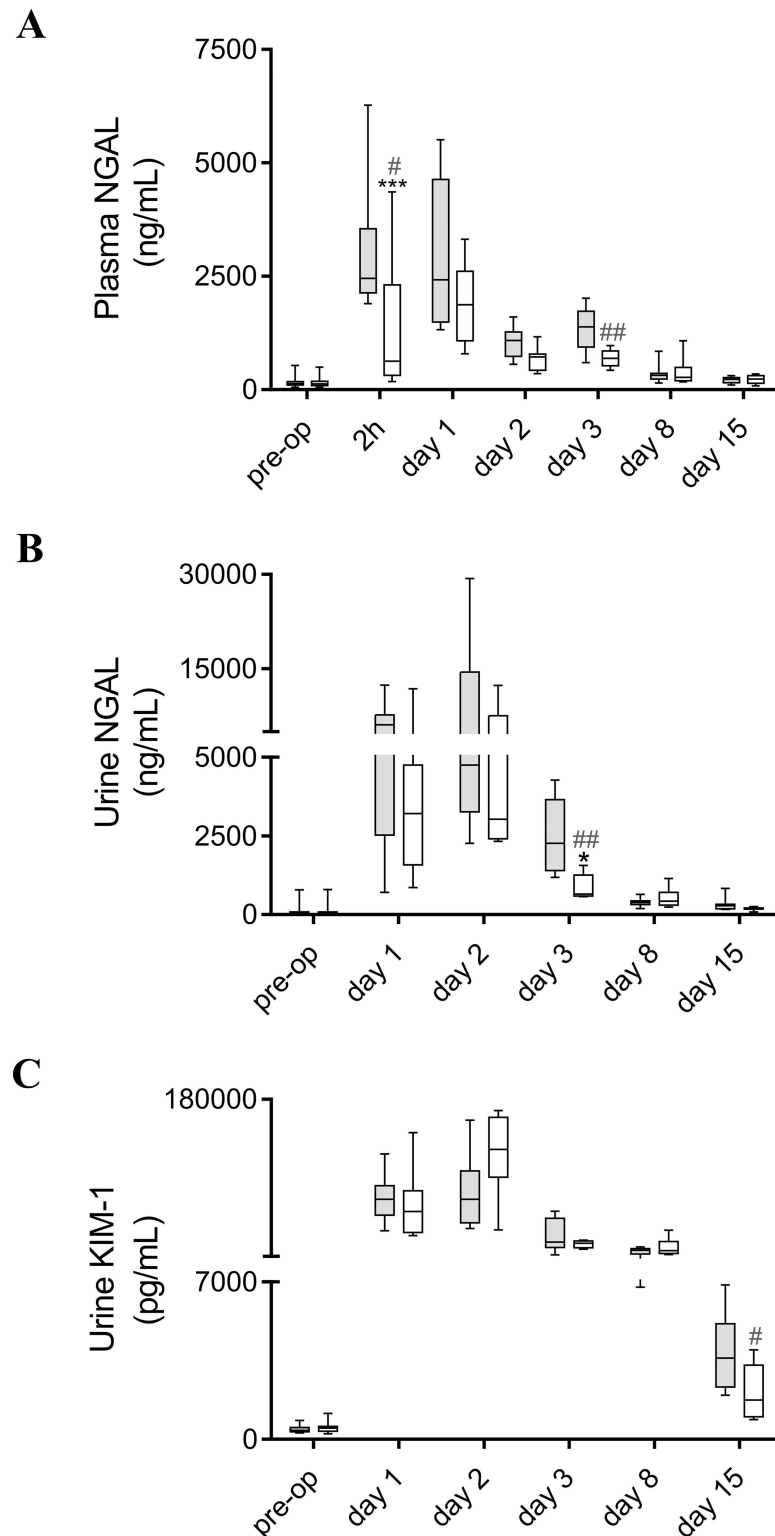


Fig 3. hAAT (80 mg/kg/day; i.p.) treatment ameliorates AKI after I/R Injury (IRI). **A.** Plasma NGAL levels at day 7 pre-op and at different time-points after IRI. **B.** Urine NGAL levels at day 7 pre-op and at different time-points after IRI. **C.** Urine KIM-1 levels at day 7 pre-op and at different time-points after IRI. Grey boxes represent the control group treated with hAlb. White boxes represent the group treated with hAAT. Two-way analysis of variance (ANOVA) followed by *Bonferroni* post-test: * $P < 0.05$, *** $P < 0.001$. Mann-Whitney U test (two-tailed) to analyze the effect of treatment at each time-point. # $P < 0.05$, ## $P < 0.01$ ($n = 6-8$ animals per group).

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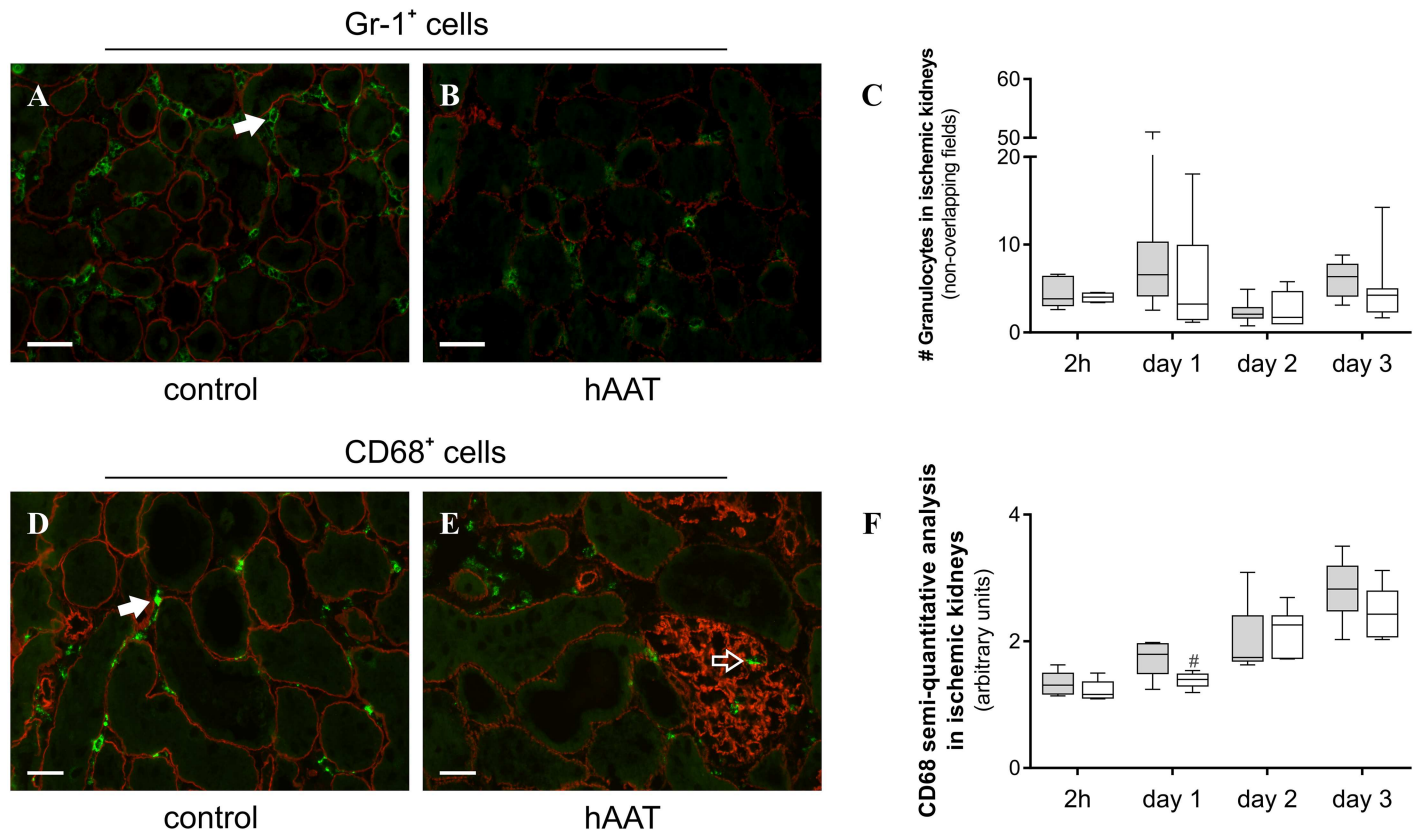


Fig 4. Effect of hAAT (80 mg/kg/day; i.p.) treatment on renal leukocyte influx at different time-points after I/R Injury (IRI). **A, B.** Immunofluorescence analysis of granulocytes in ischemic kidneys. Kidney sections were stained with a specific anti-Gr-1 antibody. **A** representative image of ischemic control mouse kidney at day 1 after IRI. **B** representative image of ischemic mouse kidney treated with hAAT at day 1 after IRI. Scale bar = 50 μ m (original magnification $\times 200$). **D, E.** Immunofluorescence analysis of macrophages in ischemic kidneys. Kidney sections were stained with a specific anti-CD68 antibody. **D** representative image of ischemic kidney of a control mouse at day 1 after IRI. **E** representative image of ischemic kidney of a mouse treated with hAAT at day 1 after IRI. Scale bar = 20 μ m (original magnification $\times 400$). Solid arrows indicate intertubular cell infiltration. The open arrow indicates cellular influx in the glomeruli. **C.** Gr-1⁺ cells were counted in non-overlapping fields and subsequently averaged. **F.** Macrophage staining was scored semi-quantitatively on a scale from 0 to 5 based on the extent of CD68 immunofluorescence staining. Grey boxes represent the control group treated with hAlb. White boxes represent the group treated with hAAT (dark green = autofluorescence of the tubuli; red = agrin; bright green = immune cells). Mann-Whitney U test (two-tailed) to analyze the effect of treatment at each time-point. [#] $P < 0.05$ ($n = 6-8$ animals per group).

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reperfusion, respectively (Fig 4, panels C and F). Immune cell influx was predominantly observed in the intertubular space with only few positive cells in the glomeruli (Fig 4, panels A, B, D and E). At day 1 post-op, there was a significant decrease in renal macrophage influx in hAAT-treated mice in comparison with control animals.

Effects of hAAT on gene expression in post-ischemic kidneys during the early phase of AKI

The acute inflammatory response triggered by I/R entails the induction of pro-inflammatory chemokines and cytokines together with the concomitant upregulation of CXC receptors. Renal gene expression of the pro-inflammatory cytokine IL-1 β , chemokines (MCP-1, MIP-1 α and KC), MGL-1, IRF5 and CXCR2 was evaluated at 2h and 1 day post-op. No significant differences were observed between experimental groups (S1 Fig).

Effect of hAAT on the recovery phase of acute tubular necrosis

Renal function was partly recovered at day 8 after 20-min ischemic insult, as can be concluded from the plasma creatinine and urea levels (S2 Fig). Histological examination of kidney tissues mirrored these findings, with partially restored brush border membranes in proximal tubules at days 8 and 15 post-op (Fig 2C). However, we also observed interstitial fibrosis on collagen staining at days 8 and 15 after reperfusion (S2 Fig, panels A-E), accompanied by an increase (relative to naïve animals) in mRNA levels of col-1 α , col-4, MMP-9 and TGF- β . Notably, no significant differences neither in the extent of renal fibrotic tissue (S2A Fig) nor in the expression of these pro-fibrotic genes (S2F Fig) were observed between hAAT-treated animals and hAlb-treated control mice.

Time-course of circulating levels of hAAT and mouse anti-hAAT antibodies

Plasma protein levels of hAAT remained high for at least 8 days after daily i.p. administration of hAAT (532.2 ± 36.81 $\mu\text{g/mL}$). After discontinuing therapy for 1 week, lower levels of hAAT were detected in the circulation at day 15 post-op (0.161 ± 0.049 $\mu\text{g/mL}$). Moreover, mouse anti-hAAT antibodies were already detected at day 8 after surgery (Fig 5A).

Discussion

The current study shows that *in vivo* administration of clinical grade hAAT improves renal function, decreases acute tubular necrosis and ameliorates AKI following experimental kidney I/R damage. Acute renal failure is a severe and potentially life-threatening clinical condition that is often the result of an extended period of renal ischemia, followed by reperfusion. In the kidney transplantation setting, the severity of AKI due to ischemic damage can impact the outcome of the graft [17]. At present, only supportive treatment is available for AKI [18–20], with no therapy available to prevent ischemic damage or to enhance its recovery. Therefore, the protective effects of hAAT observed in our murine model of bilateral renal I/R injury are quite encouraging since it could reduce the incidence of delayed graft function in kidney-transplanted patients and improve the management of acute renal failure in a variety of non-transplant conditions.

In our study, we assessed the effects of daily hAAT administration at different time-points after renal I/R injury. Although endogenous AAT levels may gradually rise as part of an acute phase response after AKI damage [8], early administration of AAT appears to be critical for optimal anti-inflammatory effects [13]. hAAT is cleared at twice the speed in mice compared to humans and therefore frequent injections were required in our experimental model. Daily hAAT administration starting 1 day before surgery until day 7 post-op resulted in sustained plasma levels of the protein. The dosage used in our study was based on earlier mouse studies, where hAAT plasma levels of 0.35–0.45 mg/mL reduced inflammation and allograft rejection in beta cell transplantation [21, 22]. Another *in vivo* study found that i.p. injection of 0.3–1.0 mg AAT protected mice from TNF α -mediated lethal response [23]. Similarly, monotherapy with 2 mg of i.p. AAT prolonged islet allograft survival [13], induced immune tolerance [24] and protected against acute myocardial I/R injury in mice [9].

The pathogenesis of kidney I/R injury is characterized by a complex interaction of renal hemodynamics, tubular damage, and consequent inflammatory responses. In the present work, we observed positive effects of hAAT on kidney morphology, macrophage infiltration and functional alterations in plasma urea levels. Next to these morphological and functional measurements, we also observed reduced levels of the kidney injury markers KIM-1 and

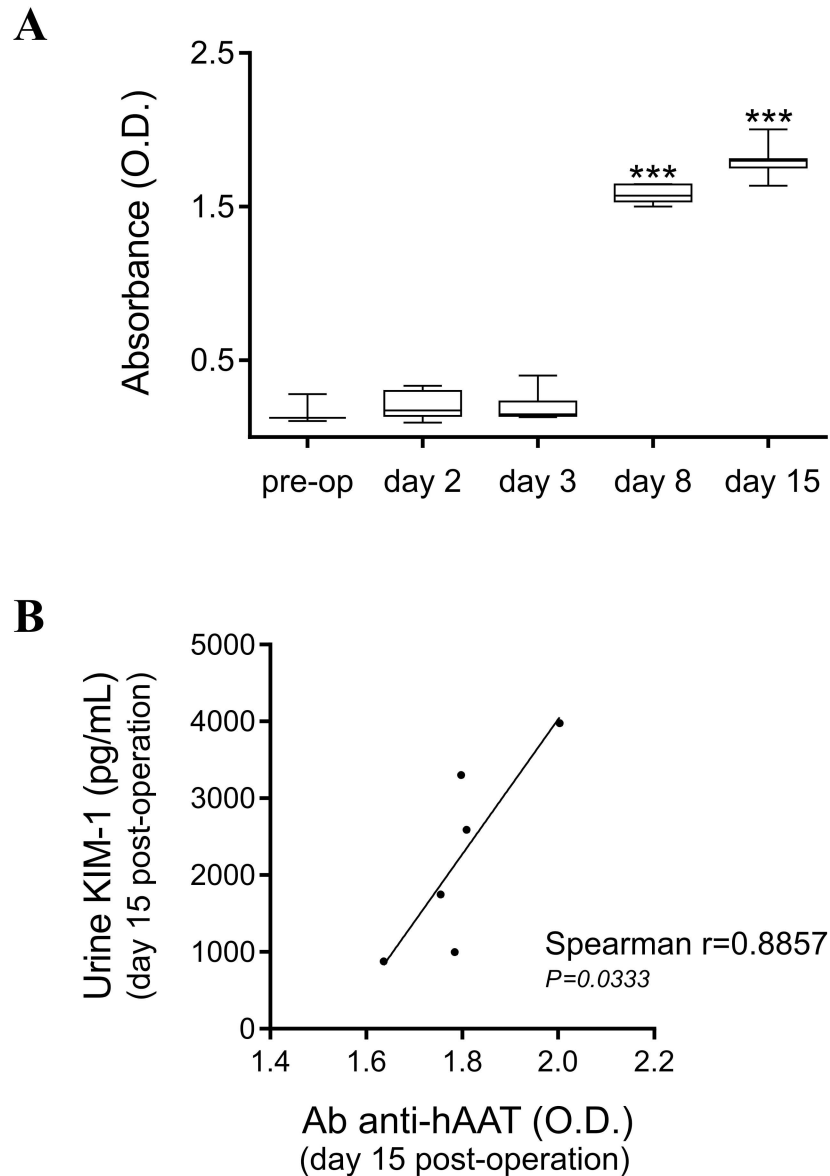


Fig 5. Mouse anti-hAAT antibody formation and its correlation with urine levels of KIM-1 during the recovery phase of renal I/R injury (IRI). **A.** Mouse anti-hAAT antibody levels in plasma were measured before surgery (pre-op) and at different time-points after IRI. One-way analysis of variance (ANOVA) followed by *Dunnnett's* post-test. *** $P < 0.001$ ($n = 4-8$ animals). **B.** Correlation between protein levels of KIM-1 in urine and mouse anti-hAAT antibodies in serum at day 15 post-op. Spearman's correlation. * $P < 0.05$ ($n = 6$ animals).

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NGAL in our experimental model. These proteins are markedly up-regulated in proximal tubule epithelial cells after ischemic damage, and can be detected in the urine of animals and patients with AKI. Furthermore, these proteins were found to be valuable noninvasive biomarkers of renal injury [25–28]. In addition, NGAL has also been investigated in serum for the early diagnosis of acute renal injury [26] and in the transplantation setting strong associations have been found between NGAL levels and biopsy-proven acute rejection [29] or acute kidney allograft dysfunction after living-donor kidney transplantation [30]. We found that KIM-1 was present at low levels in urine of healthy mice, but increased dramatically in urine of post-

ischemic animals, reaching a maximum within 24–48h post-op. In agreement with previous studies reporting maximal expression of NGAL at an earlier time-point [26, 27], this protein was up-regulated more than 100-fold in urine and more than 10-fold in plasma, peaking at 2–24h after reperfusion. There was a strong reduction in the early rise in NGAL levels in plasma ($P < 0.001$). Levels of urine NGAL and KIM-1 also significantly decreased in hAAT-treated mice during the acute and recovery phase of the I/R injury, respectively. Although NGAL and KIM-1 are both early injury markers, NGAL is longer detectable and a better and more sensitive candidate identifying injuries of the renal tubular system over a wide range of clinical conditions [31]. In mice, these early AKI biomarkers are preferred over creatinine which is not considered the most reliable measure of renal damage in this rodent species. The influence of tubular creatinine excretion on creatinine clearance is known to be larger in mice than in humans, questioning the accuracy of creatinine for determining renal function in these animals [32]. Moreover, although in clinical practice diagnosis of acute renal failure is currently performed by creatinine measurement, it is not a reliable indicator in the setting of acute changes in kidney function [33]. For instance, creatinine levels are likely not to vary until around 50% of kidney function has already been lost and this protein does not give an accurate picture of kidney function until an unfluctuating stage is achieved, which could take approximately various days. Furthermore, creatinine levels can be misleading since it depends on several factors including, but not limited to, muscle mass and metabolism, overall body weight, nutrition and the hydration status of the individual.

Renal tubular epithelial cells that are present in the oxygen-sensitive region of the outer medullar region are highly susceptible to ischemic injury [34]. In the histological analysis of 20-min ischemic kidney, the appearance of casts and debris deposition in the tubular lumen confirmed acute tubular necrosis within 1 day after I/R injury. hAAT treatment ameliorated these renal pathological manifestations. This decrease in tubular damage is most likely the result of less infiltration of innate effector cells and direct inhibition by hAAT of proteolytic enzymes that contribute to the development of injury.

After restoration of the blood flow, ischemic lesions become worse by the reintroduction of oxygen and leukocytes. Neutrophils adhesion to activated endothelium and accumulation in the ischemic kidney has been observed in both animal models and in biopsies from patients with AKI [35, 36]. Neutrophil recruitment takes place as early as 30 minutes after reperfusion, and is normally mediated by adhesion molecules such as iCAM-1, selectins and CD11/CD18 [37, 38]. However, the exact role of neutrophils in renal I/R is not well-understood and results so far have been contradictory. Inhibition of neutrophil infiltration was found to be effective in reducing experimental renal injury after I/R [37]. However, this finding could not be confirmed in another *in vivo* study [39]. Monocytes migrate to injured tissues where they differentiate into either resident mature macrophages (M1) or dendritic cells. Macrophages invade mouse renal tissue within 1 hour after reperfusion, and persist for several days [40]. During the early phase of kidney I/R, large amounts of reactive oxygen and nitrogen intermediates and pro-inflammatory cytokines (e.g. IL-1 β and TNF- α) are produced by M1 macrophages and drive a polarized Th1 immune response which contributes to injury. In mice, depletion of kidney and spleen macrophages prior to renal I/R injury prevented AKI, whereas adoptive transfer of macrophages reversed this effect [41]. In order to evaluate the effect of hAAT treatment on leukocyte influx, we analyzed the amount of macrophages and granulocytes in the post-ischemic kidneys of control and hAAT-treated mice. As previously described [42], granulocytes infiltrated the interstitial space within the first hours after 20-min ischemic period. Interstitial granulocytes peaked at 24h post-op, subsequently receding to baseline levels. On the contrary, macrophages gradually infiltrated the intertubular space over the course of the first 3 days after kidney reperfusion. Although the inhibitory effect of hAAT on neutrophil

migration is well documented [13, 43], levels of early pro-inflammatory mediators and neutrophil influx were increased by hAAT soon after peritonitis in a recent *in vivo* study [44]. In our experimental conditions, hAAT did not have a significant effect on neutrophil recruitment in post-ischemic kidneys, most likely due to the inability of hAAT to inhibit renal iCAM expression (S3 Fig). In contrast, macrophage influx significantly decreased in hAAT-treated animals at day 1 post-op. A similar inhibitory effect of hAAT on macrophage infiltration and function has been described in different inflammatory models [13, 22].

The kidney has strong regenerative capacity after injury. In contrast to most other organs such as heart and brain, the post-ischemic kidney is able to completely restore its function and structure after a mild AKI. However, when the injury is more severe, AKI can lead to incomplete tubular regeneration, proliferation of fibroblasts, chronic tubulointerstitial inflammation and excessive build-up of extracellular matrix, with progression to chronic disease [45]. Therefore, the fibrotic process occurring after acute tubular injury can have severe clinical consequences [46, 47]. TGF- β superfamily members have been extensively linked to renal fibrosis [48] since they can control the transcription of genes related to fibrotic processes including those that encode collagens. MMP-9 is also known to be involved in various inflammatory and chronic kidney diseases [49], especially in obstructive nephropathy [50]. KIM-1 is another such promoter of kidney fibrosis, which can link acute renal damage to chronic kidney disease (CKD) [51]. KIM-1 levels are enhanced in CKD [52–54], and is expressed in fibrotic and inflamed tissue [55]. Interestingly, in human allografts KIM-1 expression correlates with interstitial fibrosis [56] and higher KIM-1 urine levels predict long-term renal graft loss [57]. Therefore, KIM-1 might play an important role in the development of progressive kidney disease. Although hAAT treatment did not have a significant effect either on renal collagen deposition or on TGF- β and MMP-9 gene expression, urine KIM-1 levels at day 15 after I/R injury were significantly decreased by this serine protease inhibitor. However, this reduction of KIM-1 was not reflected by a reduction of maladaptive kidney repair in our experimental model. The lack of difference in renal fibrosis indicates that the effect of hAAT on renal injury in our experimental conditions was modest, and short-lived due to antibody formation against hAAT in mice.

Similar to what has been observed in previous studies [13, 58], repeated i.p. injections of hAAT induced a strong humoral immune response against this human protein in C57Bl/6 mice. Mouse anti-hAAT antibodies were already detected at day 8 post-op. In mice, hAAT activity is limited *in vivo* as it leads to rapid anti-hAAT antibody production after hAAT administration [13]. The lack of effect of hAAT on adverse renal remodeling could be explained by the early anti-hAAT antibody formation in our experimental setting. Interestingly, we observed a positive correlation between anti-hAAT antibody formation and urine KIM-1 levels at day 15 after kidney reperfusion (Fig 5B). Positive effects of hAAT on adverse tissue remodeling have been described previously in a mouse model of ischemic myocardial injury using the same dose regime [9]. However, in that study CD1 mice were used and anti-hAAT antibody formation was not evaluated.

Our study provides evidence of the protective effect (albeit modest) of hAAT on renal I/R injury in mice and supports the potential of this natural protein to ameliorate ischemic and inflammatory conditions. We found a significant decrease in plasma NGAL and macrophage infiltration during the first 24 hours, and a significant decrease in plasma urea levels and tubular necrosis at 48 hours. This supports a protective effect of hAAT demonstrated on four biological levels (inflammatory cell infiltration, release of tubular cell damage marker, reduced glomerular filtration, and histological injury). These differences were statistically significant despite the small number of animals used and substantial variation. However, the observed effect size was limited and one could speculate that a longer ischemia time, causing more severe

damage, would have given a more marked result. Therefore, the clinical relevance of our findings should be evaluated carefully. Given the amount of AAT required for our experiments, it was not feasible to extract such a quantity from mouse plasma nor to produce recombinant mouse AAT. In contrast, AAT purified from pooled human plasma is already commercially available as a therapeutic agent and approved at a dose of 60 mg/kg weekly by the Food and Drug Administration for replacement therapy [59]. The remarkably favorable 20-year safety profile of hAAT [60] has led to an expansion of its experimental use, highlighting the potential of this natural occurring protein as an anti-inflammatory and immunomodulatory agent. Recently, a recombinant form of AAT which appears to have stronger anti-inflammatory properties has become available [61]. Currently, clinical trials with hAAT are performed in acute graft-versus-host disease (GvHD), diabetes mellitus type I, and acute myocardial infarction. Preliminary results from trials in type 1 diabetes patients indicate that hAAT was safe and well-tolerated in pediatric subjects [62], and improved β -cell function reducing the need for insulin after hAAT treatment in recently diagnosed patients [63]. In addition, administration of Prolastin[®] in patients with ST-segment elevation myocardial infarction was also well-tolerated and decreased the acute inflammatory response [64]. The short-term protection against I/R injury provided by hAAT in our experimental model could positively impact graft outcome after organ transplantation. New therapies to reduce I/R injury of allografts are especially welcome when they are devoid of side effects. hAAT is a natural agent with proven safety in the treatment of patients who are AAT deficient. Further studies are warranted to confirm the beneficial effects of hAAT therapy in humans which would support the design of a clinical trial with hAAT in settings of renal I/R injury such as kidney transplantation.

Supporting information

S1 Fig. Effect of hAAT (80 mg/kg/day; i.p.) treatment on renal gene expression after I/R Injury. Relative mRNA expression of IL-1 β , MGL-1, MIP-1 α , MCP-1, IRF5, CXCR2 and KC in post-ischemic kidneys at 2h and day 1 post-op. Relative gene expression expressed as Rq (fold change) = $2^{-\Delta\Delta C_t}$. Grey boxes represent the control group treated with hAlb. White boxes represent the group treated with hAAT. Mann-Whitney U test (two-tailed) ($n = 6-8$ animals per group).
(PDF)

S2 Fig. Effect of hAAT (80 mg/kg/day; i.p.) treatment on kidney fibrosis after I/R Injury (IRI). **A.** Score of fibrotic area in post-ischemic kidneys at day 8 and 15 post-op. Quantification of collagen-stained area (μm^2) was performed in non-overlapping fields ($\times 200$ magnification) throughout 2 different regions of the kidney using the image software KS 400. Two-way analysis of variance (ANOVA) followed by *Bonferroni* post-test. **B-E.** Picro-Sirius Red-stained frontal sections of mouse kidney. **B, D** representative images of ischemic kidney of a control mouse at different time-points after IRI. **C, E** representative images of ischemic kidney of a mouse treated with hAAT at different time-points after IRI. OC = outer cortex; CMJ = cortico-medullary junction. Scale bar = 100 μm (original magnification $\times 100$). **F.** Relative mRNA expression of col-1 α , col-4, MMP-9 and TGF- β in post-ischemic kidneys at day 8 and 15 post-op. Relative gene expression expressed as Rq (fold change) = $2^{-\Delta\Delta C_t}$. Grey boxes represent the control group treated with hAlb. White boxes represent the group treated with hAAT. Mann-Whitney U test (two-tailed) ($n = 6-8$ animals per group).
(PDF)

S3 Fig. Effect of hAAT (80 mg/kg/day; i.p.) treatment on protein iCAM-1 expression in post-ischemic kidneys during the early phase of I/R Injury. Kidney sections were stained

with a CD54 monoclonal anti-iCAM-1 antibody (eBioscience, dilution 1:75). **A.** Representative image of ischemic control mouse kidney. **B.** Representative image of ischemic mouse kidney treated with hAAT. Scale bar = 50 μm (original magnification $\times 200$; dark green = auto-fluorescence of the tubuli; bright green = iCAM-1 protein expression).
(PDF)

S1 Table. Sequence of primers.
(PDF)

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