New approaches to decrease ischemia/reperfusion injury during kidney transplantation

Background:

The prevalence of end-stage renal disease (ESRD) worldwide is greater than ever; the number of patients increased ten times in the past twenty years. Even in Hungary the prevalence of ESRD increases by 6-7% every year. Kidney transplantation (KTx) is a life-saving treatment for ESRD patients.

Outcome of KTx is determined by a number of different issues, advances in immunology and improvements of surgical technique provide almost 95% graft survival for the first year; however delayed graft dysfunction is still the main problem affecting long-term kidney survival. Renal ischemia/reperfusion injury (IRI) is a major obstacle that contributes to delayed graft function or graft failure. Organs from expanded criteria donors are even more susceptible to IRI.

Aims:

The mechanism of IRI is clearly multifactorial, which remains largely unresolved and effective therapies are still lacking. Based on literary data we hypothesized that the molecular chaperone sigma-1 receptor (S1R) might be protective against renal IRI via the activation of brain derived neurotrophic factor (BDNF) and therefore might be beneficial during KTx. There is no data about the expression and function of S1R in the kidney to-date, and the role of BDNF and its polymorphism associated with IRI and graft survival have not been explored either.

Therefore, investigating these molecular pathways in the development of renal IRI, and in KTx, and finding new therapeutic potential of S1R agonists or BDNF is an absolute must and these were the main aims of our proposal.

Results:

I. NON-CLINICAL EXPERIMENTS

1. S1R is expressed on proximal tubular cells and is activated by agonists

To the best of our knowledge we were the first to detect S1R on proximal tubular cells, where the receptor was activated by endogenous agonist estrogen as well as exogenous agonist FLU. S1R showed a scarce, perinuclear localization in control cells, but was detected everywhere intracellularly and also in nuclei after estrogen or FLU treatment (Fig. 1.). S1R activation induced signaling pathways which resulted in HSP and nitric oxide production in proximal tubular cells (graphs not shown, see manuscripts for details).
Detailed analysis of these molecular pathways was performed in *in vivo* experiments.

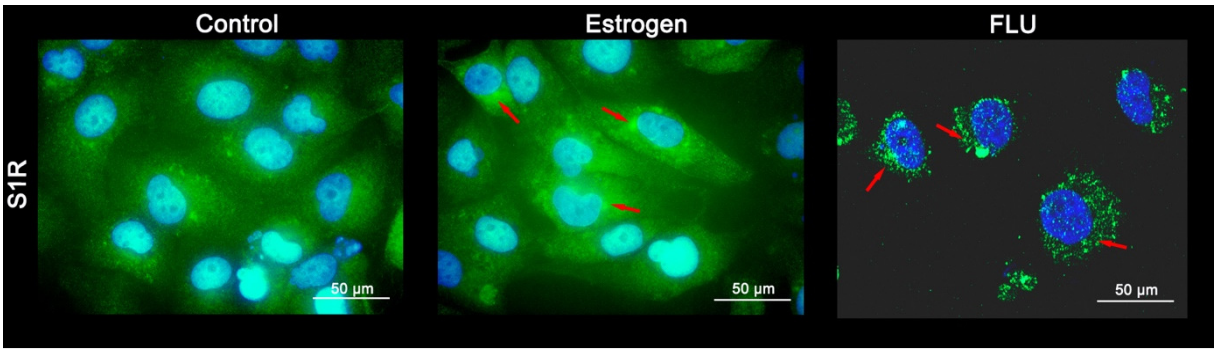

Fig. 1. Sigma-1 receptor (S1R) is activated by estrogen and FLU and translocates in proximal tubular cells. Representative images of fluorescent immunohistochemistry staining of Control; estrogen and FLU-treated HK2 cells. Anti-S1R (green); nuclei (blue); 100x magnification; scale bar=50 µm.

2. S1R expression in the kidney

S1R expression was most prominent in the renal cortex but it is also present in the medulla and papilla (Fig. 2. A-B) as shown by 3,39-diaminobenzidine (DAB) staining, fluorescent immunohistochemistry and Western blot. The next step was to prove that S1R is localized in the proximal tubules. Co-staining kidney sections with brush border membrane–specific periodic acid–Schiff (PAS) and anti–S1R DAB revealed that S1R is definitely expressed in proximal tubules but not in glomeruli (Fig. 2. C).

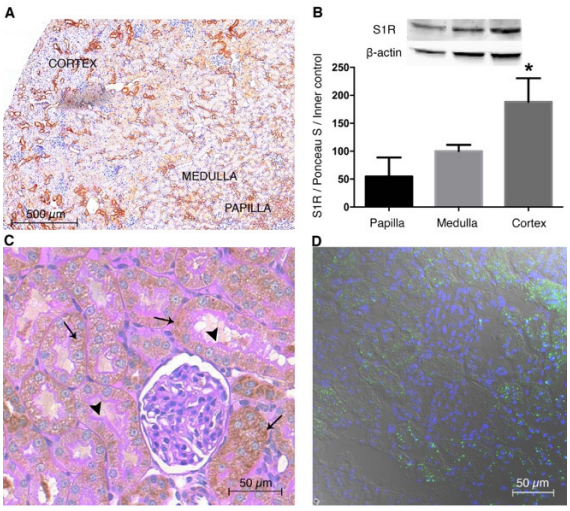

Fig. 2. S1R is predominantly expressed in the renal cortex. (A) Representative rat kidney section stained with anti-S1R (brown; 6X DAB). Scale bar, 500 µm. (B) Immunoblot for renal S1R. Bars indicate means+SEMs; data were analyzed by one-way ANOVA with Bonferroni multiple comparison test. *P<0.05 versus papilla and medulla (n=6 per group). (C) Representative kidney section developed with peroxidase anti-S1R (brown) and counterstained with PAS (pink). Thin black arrows show S1R in proximal tubules but not in glomerulus. Black arrowheads point to intact, PAS–stained brush borders of proximal tubules. Scale bar, 50 µm. (D) Representative image of fluorescent immunohistochemistry double staining of rat kidney for nuclei (blue) and S1R (green). Scale bar, 50 µm.

3. Endogenous S1R agonists estrogen and DHEA mitigate ischemic renal injury

The influence of S1R agonist sex hormones on the heat shock pathway during IRI was tested *in vivo*. Renal ischemia was accomplished by cross-clamping the left renal hilus for 50 min with an atraumatic vascular clamp. The contralateral kidney was removed and samples were collected 2 hours (T2) and 24 hours (T24) after reperfusion.

Both serum creatinine and BUN levels were massively increased at T24 in all groups reflecting the development of AKI. At T24 serum creatinine levels were less elevated in females and DHEA treated males, while BUN levels were lower in all groups vs. males (Fig. 3.). IRI caused extensive tubular necrosis, hyalinization and interstitial lesions, which were less prominent in female and DHEA-treated male rats.

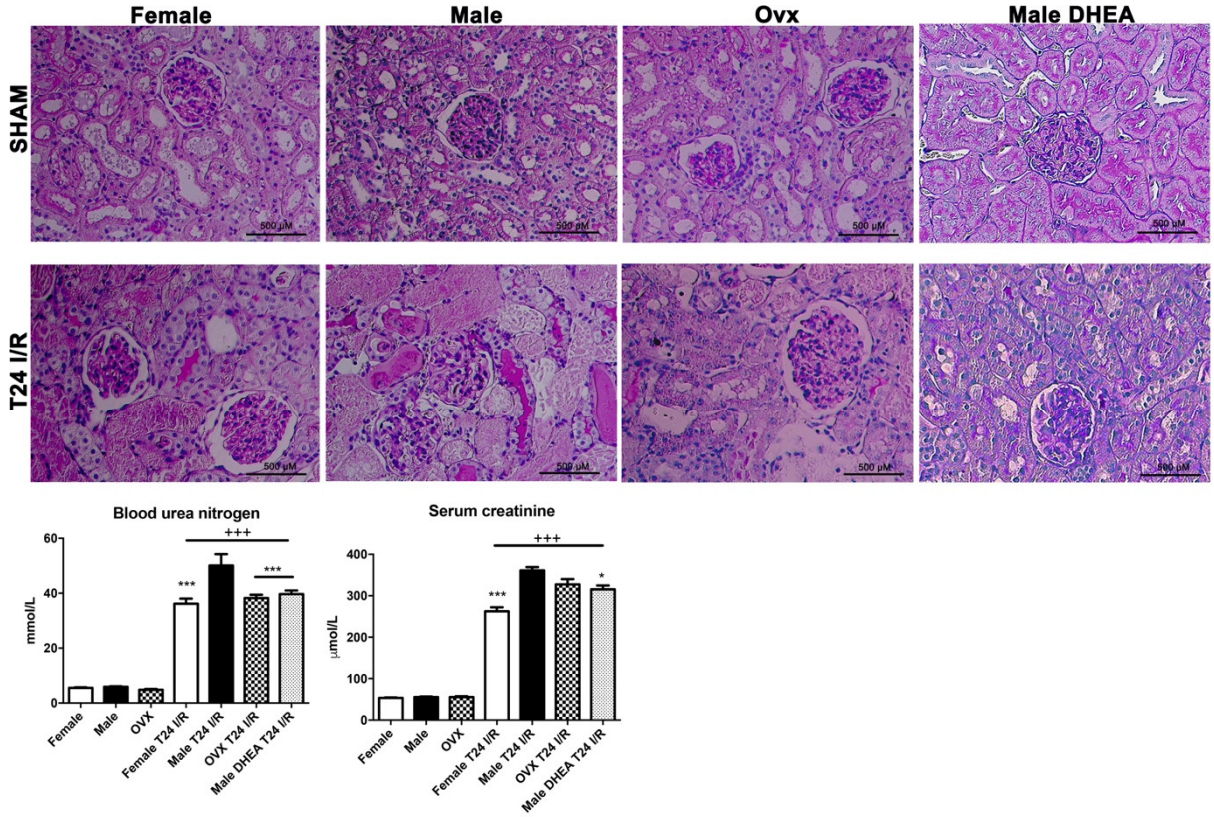

Fig. 3. Postischemic renal lesions and functional decline are milder in female and DHEA-treated male rats. Representative periodic acid-Schiff-stained kidney sections show structural damage at 24 hours (T24 I/R) after reperfusion in Female, Male, ovariectomized female (Ovx) and DHEA-treated male rats. Magnification: ×400, scale bar=500 µm. $^{+++}$P<0.001 *vs.* Female, Male or Ovx SHAM resp.; *P<0.05 *vs.* T24 I/R Male; ***P<0.001 *vs.* T24 I/R Male; n=8 per group; bars indicate means+SEMs.

4. S1R and HSP signaling is more prominent in females after IRI

Renal S1R and phospho-Akt (pAKt) levels remained unaltered after IRI in males and Ovx, but there was a marked increase in females at T2. Proteins possibly activated by S1R showed similar dynamics. Baseline HSF-1, HSP-72, HSP-27 and NKA protein levels were higher in females and elevated further after the insult (Fig. 4.). Protein levels of the Ovx group followed the levels of males in most cases confirming the role of female sex hormones.

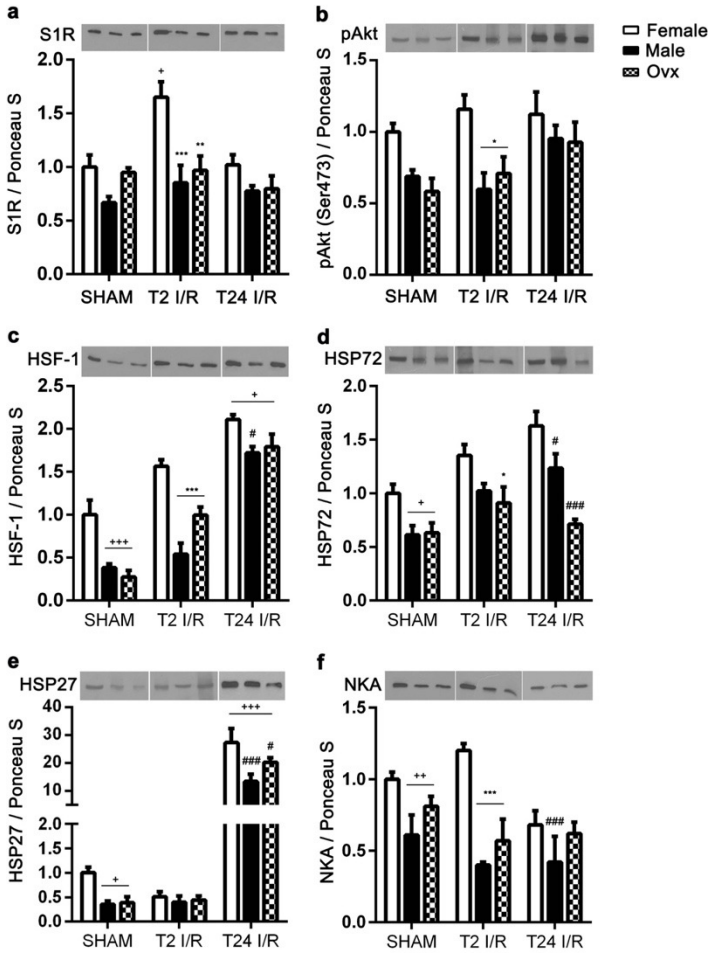

Fig. 4. Female sex activates the production of renal S1R, pAkt, HSF-1, HSP72, HSP27 and Na^+, K^+-ATP-ase (NKA) protein following IRI.
(a) Sigma-1 receptor (S1R), (b) phospho-Akt (pAKt) (Ser473), (c) heat shock factor-1(HSF-1), (d) heat shock protein 72 (HSP72), (e) heat shock protein 27 (HSP27), and (f) Na^+, K^+-ATP-ase (NKA) protein levels at 2 (T2) and 24 (T24) hours after reperfusion in female, male and ovariectomized female (Ovx) rats. $^{+}$P<0.05 *vs.* Female SHAM; $^{++}$P<0.01 *vs.* Female SHAM; $^{+++}$P<0.001 *vs.* Female SHAM; *P<0.05 *vs.* T2 I/R Female; **P<0.01 *vs.* T2 I/R Female, ***P<0.001 *vs.* T2 I/R Female; $^{\#}$P<0.05 *vs.* T24 I/R Female; $^{\#\#\#}$P<0.001 *vs.* T24 I/R Female; n=8 per group; bars indicate means+SEMs.

5. FLU pretreatment improves survival as well as kidney function after IRI

To further prove the crucial role of S1R in IRI, we proceeded with FLU, which has a much higher affinity to S1R than estrogen or DHEA (Fig. 5.). As primary end point we showed 30% postischemic survival only with FLU treatment; while renal function was more preserved in FLU vs. vehicle treated rats. In parallel *in vivo* two-photon microscopy revealed that structural damage is also mitigated in FLU-treated rats following IRI (Fig. 6.).

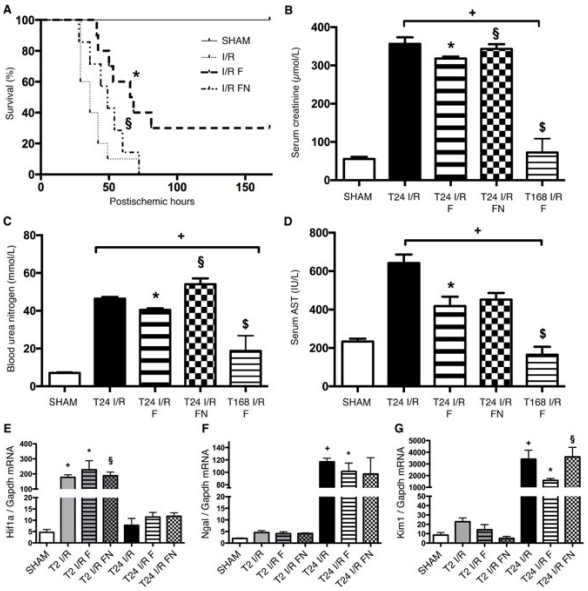

Fig. 5. FLU pretreatment is protective against renal IRI. (A) Rats were pretreated with isotonic saline (I/R), FLU (I/R F), or FLU and S1R antagonist NE100 (I/R FN). Postischemic survival was followed for 7 days; n=8 per group. *P<0.001 versus I/R; §P<0.001 versus I/R F. (B) Serum creatinine levels after 24 hours of reperfusion and after 168 hours of reperfusion in FLU-treated (T168 I/R F) rats. (C) BUN levels. (D) Serum AST levels. (E) Renal Hif-1a, (F) Ngal, (G) Kim1 mRNA expressions normalized to Gapdh expression. *P<0.05 versus T24 I/R; +P<0.05 versus sham; §P<0.05 versus T24 I/R F; $P<0.05 versus T24 I/R; n=8 per group; bars indicate means+SEMs.

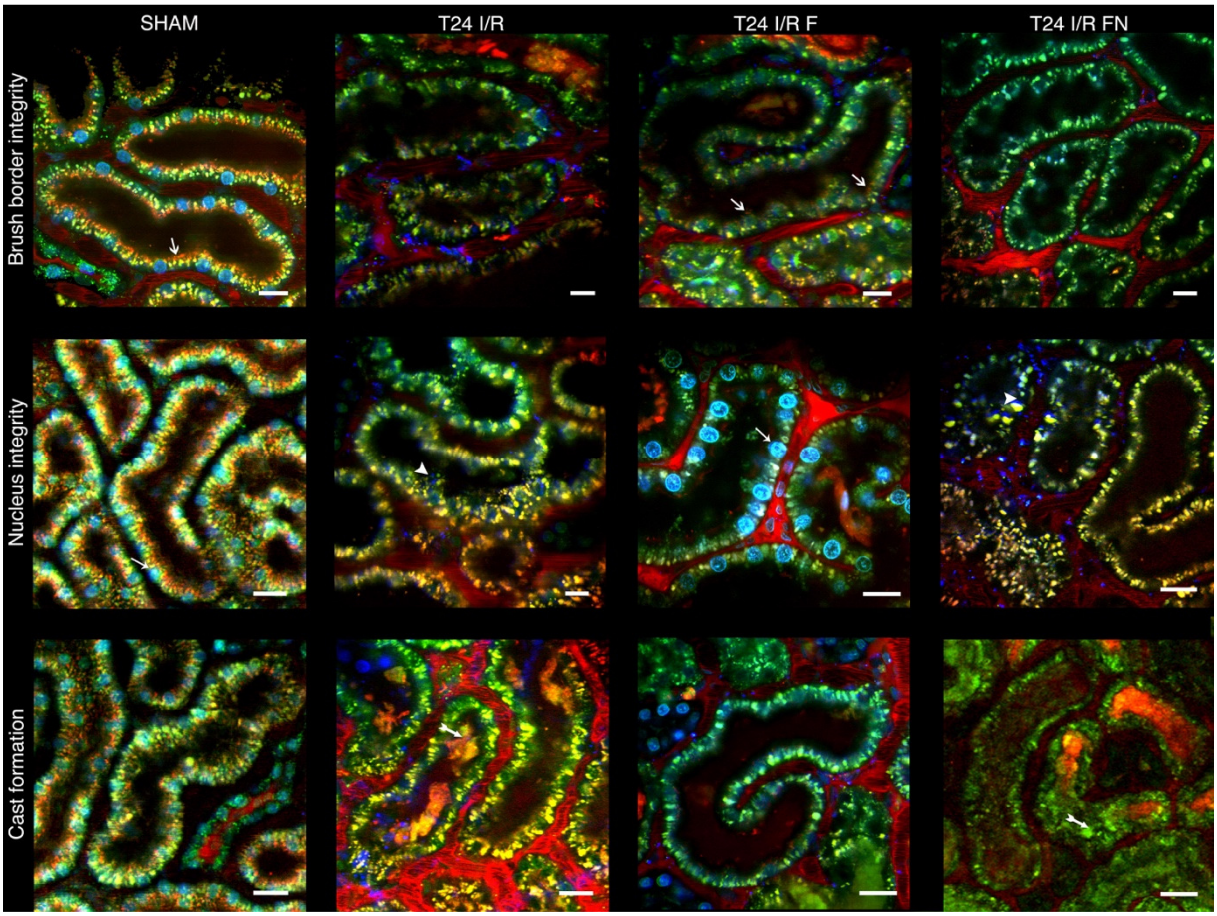

Fig. 6. FLU mitigates structural damage after IRI.
Representative intravital two–photon images of the rat kidney after 24 hours of reperfusion. White arrows show orange–colored Texas Red staining of intact brush borders. Nuclei stained with Hoechst 33342 appear in blue. Thin white arrows point to intact nuclei. White arrowheads show severely damaged, disintegrated nuclei. In bottom row, two–tailed white arrows show extensive necrotic cast formation. n=3 per group; scale bar, 25 μm.

6. FLU induces S1R-mediated vasodilatative NOS production in the kidney

Parallel with increased peNOS and NO production FLU pretreatment increased peritubular capillary diameters (Fig. 7. A-C). Proteins of the signaling pathway were evaluated as well. FLU increased pAkt, peNOS and nNOS protein levels after IRI (Fig. 7. D-F).

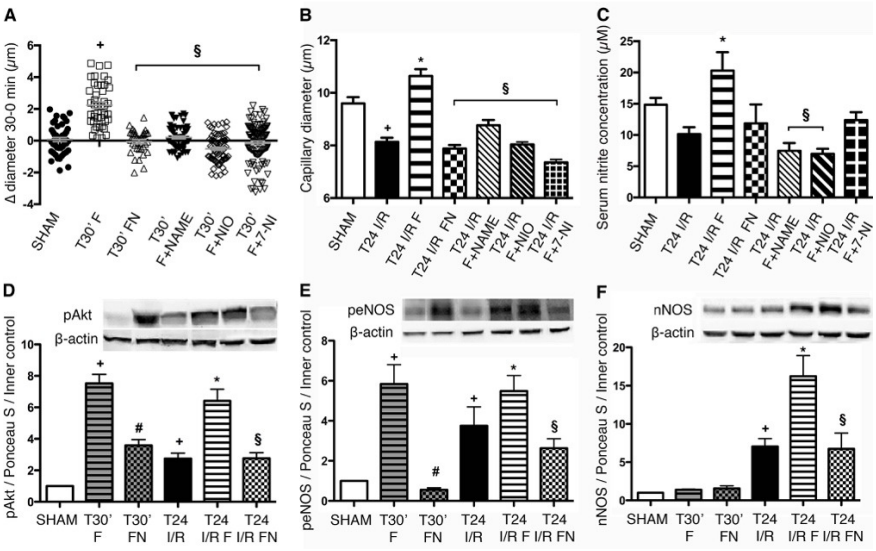

Fig. 7. FLU induces S1R–mediated NOS production and vasodilation in the rat kidney.
(A) Changes in capillary diameters 30 minutes after FLU (T30' F), FLU + NE100 (T30' FN), FLU+nonselective NOS blocker N-v-Nitro-L-arginine methyl ester (T30' F+NAME), FLU+selective eNOS blocker N-5-(1-Iminoethyl)-L-ornithine dihydrochloride (F+NIO), and FLU+selective nNOS blocker 7-Nitroindazole (F+7-NI) treatment in sham-operated rats. +P<0.05 versus sham; §P<0.05 versus T30' F; n=3 per group. (B) Capillary diameters after 24 hours of reperfusion. (C) Serum nitrite concentration of rats after 24 hours of reperfusion. *P<0.05 versus T24 I/R; +P<0.05 versus sham; §P<0.05 versus T24 I/R F n=3 per group. (D) pAkt (Ser473), (E) peNOS (Ser1177), and (F) nNOS expressions. *P<0.05 versus T24 I/R; +P<0.05 versus sham; #P<0.05 versus T30' I/R F; §P<0.05 versus T24 I/R F; n=5–7 per group; bars indicate means+SEMs.

7. FLU increases BDNF production after IRI

BDNF and its signaling pathways play a pivotal role in the mitigation of cerebral IRI by reducing neuronal apoptosis and neuroinflammation. Although the neuroprotective effect of BDNF is well known in brain IRI, there is no data concerning the relation of BDNF and IRI in the kidney.

Both pro (proBDNF) and mature BDNF (mBDNF) levels were reduced in IRI, while FLU treatment elevated both forms in the kidney (Fig. 8.).

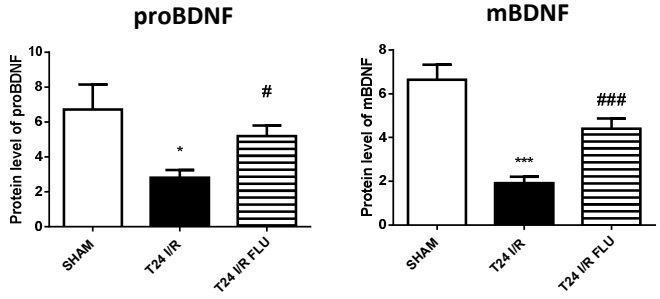

Fig. 8. Pro and mature (m) BDNF levels are normalized by FLU following IRI.
*p<0.05 or ***p<0.001 *vs.* SHAM, #p<0.05 or ###p<0.001 *vs.* T24 I/R; n=7-8 per group; bars indicate means+SEMs.

8. S1R agonists improve renal function in a rat autotransplantation model

KTx offers better quality of life and reduced costs compared to dialysis, but shortage in donor organs is a limiting factor. Graft survival and function are highly dependent on the extent of IRI during KTx, therefore we aimed to develop a preservation solution which minimizes ischemic graft damage in order to improve KTx outcomes and to increase the number of organs suitable for KTx. In a rat model of autotransplantation (ATx) grafts were stored in cold preservation solution containing S1R agonists (FLU; SA-4503 (SA)) before the transplantation procedure.

Both S1R agonists improved kidney function and mitigated tubular damage following ATx (Fig. 9.).

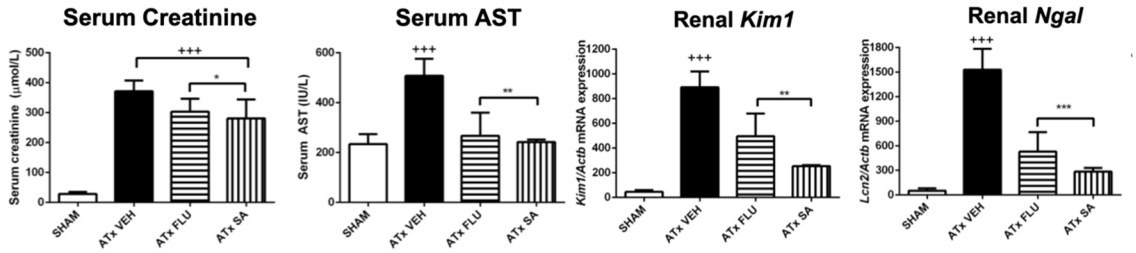

Fig. 9. A. S1R agonists FLU and SA mitigate renal damage after ATx.
Serum creatinine, serum aspartate aminotransferase (AST) levels and *Kim1*, *Ngal* mRNA expressions 24 hours after ATx. +++p<0.001 *vs.* SHAM; ∗p<0.05 *vs.* ATx VEH; ∗∗p<0.01 *vs.* ATx VEH; ∗∗∗p<0.001 *vs.* ATx VEH; n=8 per group; bars indicate means+SEMs.

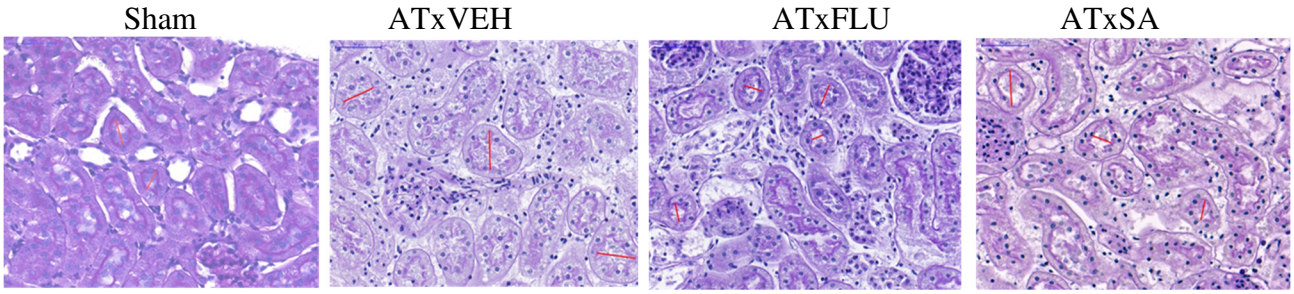

Fig. 9B. S1R agonists FLU, SA mitigate renal tubular dilatation after ATx.

9. FLU increases BDNF levels in ATx

Mature BDNF (mBDNF) levels decreased after ATx, while pro BDNF (proBDNF) remained unchanged. ProBDNF showed a rising tendency, moreover mBDNF was markedly increased after FLU treatment (Fig. 10.).

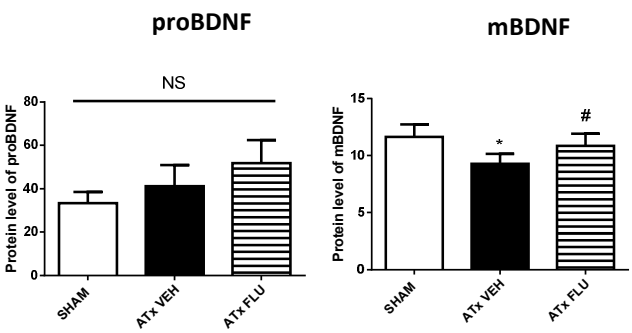

Figure 10. Pro and mature BDNF levels following ATx.
*p<0.05 *vs.* SHAM, #p<0.05 vs. ATx VEH; n=8 per group; bars indicate means+SEMs.

10. FLU abates apoptotic and inflammatory processes following ATx

BDNF is known to reduce apoptosis and inflammation in cerebral IRI. We found similar results in ATx, where FLU abated apoptotic processes, induced anti-apoptotic *Bcl2* expression and mitigated inflammatory cytokine expression in the kidney (Fig. 11.).

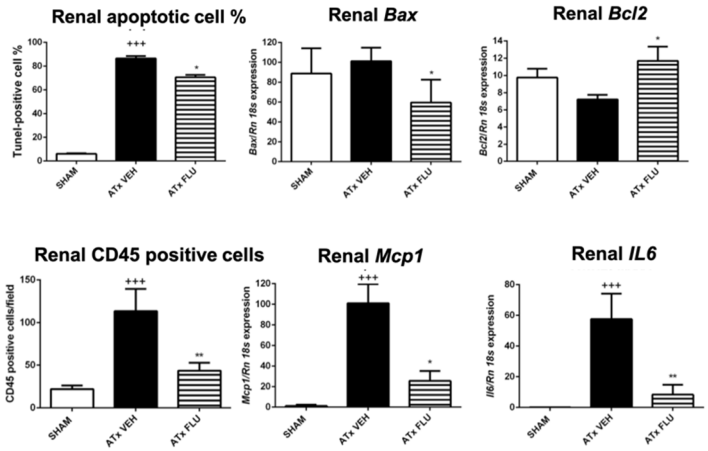

Fig. 11. FLU abates apoptotic and inflammatory processes following ATx.
Apoptotic cells counted on Tunel-stained sections; pro-apoptotic *Bax* and anti-apoptotic *Bcl2* mRNA expressions; number of CD45+ leukocytes; inflammatory cytokine *Mcp1* and *IL6* mRNA expressions. +++p<0.001 *vs.* SHAM; ∗p<0.05 *vs.* ATx VEH; ∗∗p<0.01 *vs.* ATx VEH; n=8 per group; bars indicate means+SEMs.

11. S1R agonists mitigate cold ischemic structural kidney damage

To test the potential protective effect of S1R agonists against cold ischemic injury, kidneys were perfused with a preservation solution containing various S1R agonists (FLU; SA-4503; PRE-087) and were stored in these solutions for various amounts of time. Structural damage of kidneys stored in solutions containing S1R agonists was milder at all time points (Fig. 12.), therefore maximum storage time and the number of transplantable grafts can be increased.

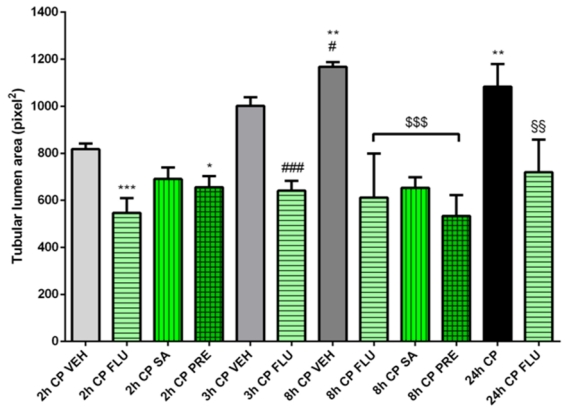

Fig. 12. Cold ischemic structural damage is mitigated by S1R agonists.
Structural damage after various times of cold perfusion (CP) evaluated on PAS-stained kidney sections. ∗p<0.05 *vs.* 2h CP; ∗∗p<0.01 *vs.* 2h CP; ∗∗∗p<0.001 *vs.* 2h CP; #p<0.05 *vs.* 3h CP; ##p<0.01 *vs.* 3h CP; $$$p<0.001 *vs.* 8h CP; §§p<0.01 *vs.* 24h CP; n=6 per group; bars indicate means+SEMs.

II. HUMAN STUDIES

The aim of our human clinical study was to explore the relationship between serum BDNF concentration, BDNF gene polymorphism in CKD and renal graft function after transplantation.

Serum BDNF, creatinine, blood urea nitrogen, and blood glucose levels were measured, hemoglobin levels and thrombocyte numbers at 1 week, 1-, 3-, 6 months, and 1-, 2 years after transplantation surgery. GFR was estimated based on the CKD-EPI formula. Urinary BDNF levels were below lower limit of detection. BDNF Val66Met polymorphism was determined via PCR-RFLP method.

Results:

We enrolled 59 ESRD (Dept. of Transplantation and Surgery, Semmelweis University) patients with average age of 54.8±12 years who received renal transplantation. Proportion of male patients was 57%. Only 3 patients received their kidney graft from a living donor, the rest were recipients of cadaveric organ donation. Cold ischemic time was 927 ± 310 min. Warm ischemic time was 54.5±39 min. Delayed graft function occurred in 5 cases. Baseline triple immunosuppression therapy consisted of calcineurin inhibitor (tacrolimus), antimetabolite (mycophenolate) or mTOR inhibitor (everolimus), and corticosteroid (prednisolone).

Until this time point, 44 patients completed the 2 years follow-up, and 6 patients were lost. In order to establish a comparable control group, we collected blood samples from 79 healthy volunteers with average age of 53.9±16 years and with male gender proportion of 52%. Same parameters were measured as from the transplanted patients (see Table below).

	Control	ESRD	After transplantation					
			1 week	1 month	3 months	6 months	1 year	2 years
Number of patients	79	59	59	58	55	54	53	44
Average age	53.9 ± 16	54.8 ± 12	54.8 ± 12	54.7 ± 12.1	54.3 ± 11.9	54 ± 11.8	55.1 ± 11.9	56.6 ± 12.1
Proportion of males	0,519	0,576	0,576	0,569	0,582	0,574	0,600	0,595
Serum BDNF level (pg/mL)	25234 ± 8494	22472 ± 7938	19667 ± 8517	23146 ± 10046	26185 ± 10965	25863 ± 9936	29594 ± 10663	26950 ± 9377
Serum creatinine level (µmol/L)	76.4 ± 14.7	641.5 ± 258.9	228.1 ± 180.5	141.3 ± 54.2	140.2 ± 58.9	139.8 ± 55.1	140.6 ± 64.6	158.5 ± 99.1
Serum carbamid level (mmol/L)	6.3 ± 5.3	17.2 ± 8.0	15.3 ± 9.5	10.1 ± 5.5	8.9 ± 4.5	9.6 ± 5.1	9.7 ± 5.3	10.6 ± 5.6
eGFR (mL/min/1.73m^2)	89.5 ± 15.9	8.9 ± 6.4	39.2 ± 23.6	48.3 ± 19.9	45.5 ± 22.3	47.5 ± 19.7	49.3 ± 19.4	45.6 ± 18.2
Hemoglobin level (g/L)	146.9 ± 12.6	119.8 ± 14.7	103.9 ± 13.3	115.9 ± 14.3	121.0 ± 23.2	134.8 ± 26.2	134.8 ± 26.2	135.9 ± 28.1
Thrombocyte number (G/L)	255.9 ± 57.3	212.2 ± 64.0	221.0 ± 89.5	224.1 ± 89.9	238.5 ± 79.1	232.6 ± 79.7	234.9 ± 62.1	217.3 ± 63.6
Blood glucose level (mmol/L)	5.2 ± 0.8	6.6 ± 2.1	5.4 ± 1.3	6.9 ± 2.9	6.8 ± 2.6	6.1 ± 1.7	6.4 ± 2.0	6.5 ± 2.4

Genotype distribution			
	Control	Transplanted	Sum
Val/Val	49	42	91
Val/Met	22	14	36
Met/Met	2	1	3
Sum	73	57	130

Genotype distribution			
	Control	Transplanted	Sum
Val/Val	0,671	0,737	0,700
Val/Met	0,301	0,246	0,277
Met/Met	0,027	0,018	0,023
Sum	1	1	1

Allele distribution			
	Control	Transplanted	Sum
Val	120	98	218
Met	26	16	42
Sum	146	114	260

Allele distribution			
	Control	Transplanted	Sum
Val	0,822	0,860	0,838
Met	0,178	0,140	0,162
Sum	1	1	1

Main findings: There was no difference in genotype or allele distribution between any of the groups. There was no correlation between serum BDNF and different genotypes either. The level of serum BDNF was lower in ESRD patients versus healthy controls (p=0.03).

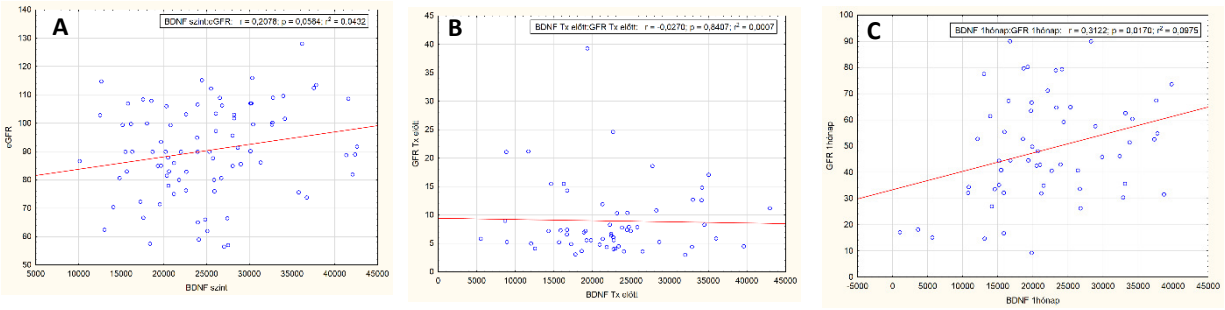

Fig. 13. Correlation of serum BDNF level with eGFR in controls (A), in ESRD patients before KTx (B) and in transplant recipients 1 month after KTx (C).

There was a weak correlation and marginal significance (p=0.056) between eGFR and serum BDNF level in controls, while in KTx recipients this correlation reached a stronger power (r2=0.0975) with a much higher significance (p=0.01), (Fig. 13.). Above median BDNF values at 1month after KTx were predictive for better graft function during the 2 obsereved years (Fig. 14).

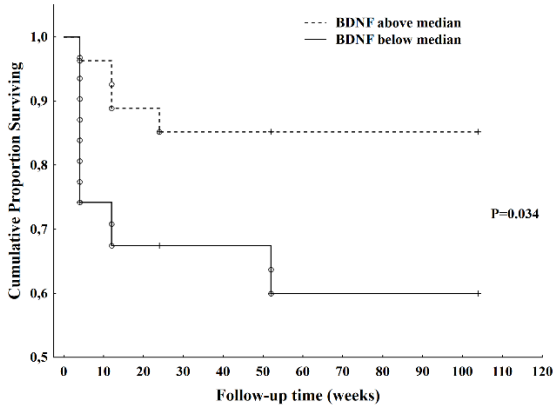

Fig. 14. Kaplan-Meier analysis revealed that in KTx group the 2-year survival with good-functioning graft (eGFR>40ml/min) is higher in those patients whose 1 month serum BDNF level is over the median BDNF concentration.

Summary:

In summary, we are the first to show that S1R agonism is protective in renal IRI essentially by improving postischemic survival and renal function and ameliorating renal structural damage. We confirmed the role of sex hormones in superior outcomes in females after renal I/R injury and propose a S1R-mediated molecular pathway which could contribute to the renoprotection females enjoy. Our results indicate that activation of S1R by E2 is protective by enhancing the heat shock response in the kidney.

Our findings have chalked out a molecular pathway of S1R–mediated renal vasodilation involving S1R translocation, activation of the Akt pathway, BDNF and NO production. In our transplant model, S1R agonist FLU substantially improved posttransplant renal function and ameliorated renal damage via activating S1R signaling in the kidney, which suggests that S1R could be an ideal target to prevent renal IRI. Our preliminary human study proposes that BDNF could be a novel biomarker of posttransplant graft function, however further clinical studies with significantly larger population are definitely needed to confirm these results.

Publication/grants: Our preclinical results as original articles have been published in peer-reviewed leading journals of nephrology (e.g. J Am Soc Nephrol) or transplantation (Transplant Int). We participated each year with posters or oral presentations at three national congresses (MANET, MTT, Mathiné) and two international ones (ERA-EDTA, ASN or ESOT). With proposals based on these results our group has been a successfull submitter of a VKE grant of 996M HUF, an ERC visiting grant of 3M HUF and STIA grants of 5M HUF.
Prizes and education: Member of the group Adam Hosszu won several scientific prizes including Imre Ulmann award of the Hungarian Transplant Association, Veritas et Virtus publication prize of the Semmelweis University and he has also been awarded with the ERA-EDTA National grant. Two of our graduate students won the best presentation prize at the Congress of Young Nephrologists and several lectures were held on the Students conference of the Semmelweis University, two of them won 1st, and three of them 2nd prize.
Two former PhD students (Adam Hosszu and Lilla Lenart) successfully defended their PhD thesis and an additional one (Zsuzsanna Antal) will defend in June 2019.