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Acute Kidney Injury Induces Remote Cardiac Damage and Dysfunction Through the galectin-3 Pathway

Brief Title: Gal-3 promotes cardiac damage after kidney injury.

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HIGHLIGHTS:

- In two different mouse models, acute kidney injury (AKI) increased Galectin-3 (Gal-3) expression and induced cardiac dysfunction, cardiac and systemic inflammation, cardiac macrophage infiltration and fibrosis.
- Cardiac consequences of AKI were dependent of the Gal-3 pathway, and were prevented using Gal-3 knockout mice or modified Citrus pectin as pharmaceutical inhibitor.
- Cardiac Gal-3 expression resulted from Bone marrow-derived immune cells recruitment after AKI.
- In critically ill patients, development of AKI is associated with increased plasma Galectin-3 level and increased biomarkers of cardiac injury and damage.

SUMMARY:

Acute kidney injury is associated with increased risk of heart failure and mortality. This study demonstrates that acute kidney injury induces remote cardiac dysfunction, damage, injury and fibrosis via a galectin-3 dependent pathway. Galectin-3 originates from bone marrow-derived immune cells. Cardiac damage could be prevented blocking this pathway.

Key words: heart failure, fibrosis, renal failure, inflammation, macrophages

Abbreviations:

AKI= Acute Kidney Injury

BM= Bone Marrow

BUN= Blood Urea Nitrogen

cTGF= Connective Tissue Growth Factor

Cr= Creatinine

eGFR= Estimated Glomerular Filtration Rate

Gal= Galectin

KO= Knock-out

HIF= Hypoxia Inducible Factor

ICAM= Intercellular Adhesion Molecule

ICU= Intensive Care Unit

IL= Interleukine

IR= Ischemia-reperfusion

KDIGO= Kidney Disease-Improving global outcome

MCP= Modified Citrus Pectin

MCP-1= Monocyte Chemoattractant Protein-1

mRNA= Messenger Ribonucleic Acid
NGAL= Neutrophil Gelatinase Associated Lipocalin
NT-proBNP= N-terminal-pro brain natriuretic peptide
SAPS= Simplified Acute Physiology Score
SOFA= Simplified Organ Failure Assessment
TGF= Transforming Growth Factor
TNF= Tumor Necrosis Factor
UUO= Unilateral Ureteral Obstruction
VCAM= Vascular Cell Adhesion Molecule
WT=Wild Type

Introduction:

Acute kidney injury (AKI) has been associated with an increased risk of mortality(1), even in patients free of previous cardiovascular comorbidities(2). Furthermore, AKI was shown to be associated with long-term cardiovascular events(3). Whether the occurrence of AKI only reflects the severity and magnitude of underlying disease or may contribute itself to remote organ injury remains uncertain. Remote cardiovascular injury may contribute to poor outcomes after AKI(4). In this line, AKI was also associated with long-term cardiovascular events. This has been described in type 3 cardiorenal syndrome (or acute reno-cardiac syndrome)(5). However, whether transient AKI can induce long-term cardiac injury remains unexplored. Cardiac fibrosis has been described as a key feature of chronic heart diseases(6). Galectin-3 (gal-3) is a lectin that specifically binds to β -galactosides(7) expressed in many tissues including the heart and kidney(8)(9). In the heart, gal-3 induction promotes myocardial fibrosis and heart failure progression(10)(11). Gal-3 has been shown to be a key player in cardiac fibrosis induction and proposed as a prognostic biomarker for chronic heart failure(7). In the kidney, gal-3 was also shown to be up regulated after AKI(12). In the present study, we hypothesized that AKI can activate gal-3-dependent pathways and promote cardiac injury. For this purpose, we explored the role of gal-3 in cardiac injury after kidney injury. Moreover, a bone marrow graft from gal-3 KO mice was used to determine gal-3 origin.

Materials and Methods:

Animals

Two to four-month old male C57Bl6/J, from Janvier laboratory and C57Bl6/J Knock Out for galectin-3 (gal-3 KO(13)) mice were used. All animals were randomized into different groups after baseline echocardiography. Methods of echocardiography, plasma assays, gene expression

analysis, protein analysis, immunostaining, cardiac fibrosis evaluation, renal macrophage isolation, cell culture and monocyte adhesion assays are detailed in supplementary file.

Renal and hind limb ischemia reperfusion injury

A right nephrectomy and left renal pedicle occlusion (25 minutes of ischemia) followed by reperfusion were performed under anesthesia (intra peritoneal injection of Ketamine: 100mg/kg and Xylazine: 20mg/kg). Right kidneys were used as controls. Sham mice underwent the same procedure excepted renal pedicle occlusion and right nephrectomy(14). To get insights into the kinetics of the kidney-heart cross talk after renal IR, mice were sacrificed at different time points after reperfusion (3h, 6h, 12h, 24h, 48h, 72h and 28 days; n=7 per group) (**Online Figure 4A**). Another mouse group was submitted to left femoral artery occlusion for 25 minutes followed by reperfusion. These mice were sacrificed 28 days after surgery (hind limb ischemia).

Bone marrow transplantation

Mice were irradiated at 10 grays (2x5 gray at 5-hour interval) with filter, with a Faxitron irradiator. Just after the second irradiation, mice were grafted with bone marrow from WT or KO. At the end of protocol two groups of chimeric mice were obtained: WT mice grafted with KO gal-3 bone marrow (WT^{KO} BM) and KO gal-3 mice grafted with WT bone marrow (KO^{WT} BM). These chimeric mice were submitted to right nephrectomy and left renal ischemia/reperfusion (IR) injury, as described previously. Sham mice underwent the same procedure except right nephrectomy and left renal ischemia/reperfusion. (**Online Figure 1B and Online Figure 2**).

Unilateral Ureteral Obstruction

A left ureteral obstruction was performed under anesthesia. The ureter was subsequently ligated in two places near the kidney. Sham mice underwent the same procedure except ureteral obstruction (**Online Figure 4C**).

Treatments

The gal-3 inhibitor Modified Citrus Pectin (MCP) was dissolved into drinking water (100 mg/kg per day). Mice were either pre-treated with MCP (IR+MCP) 3 days before surgery and during the time of reperfusion or treated one day after surgery (IR+MCP d+1).

Human Cohort

The association between AKI, gal-3 and cardiac injury was explored in the French and European Outcome Registry in Intensive Care Units (FROG-ICU) Cohort (NCT01367093). This study was an international observational study, including consecutive critically ill patients admitted to 23 ICUs who received mechanical ventilation and/or vasopressors. The protocol has been previously described elsewhere(15). In this sub-analysis, patients with chronic kidney disease were excluded. The study population included 1110 patients discharged from ICUs with neutrophil gelatinase associated lipocalin (NGAL) available on ICU admission and galectin-3 at ICU discharge. AKI was defined by the Kidney Disease-Improving global outcome (KDIGO) definition (clinical AKI) or NGAL >150 ng/ml at ICU admission (sub-clinical AKI)(16)(17). Plasmatic levels of cardiac injury/stress biomarkers were measured at ICU discharge (Plasma gal-3, N-terminal-pro brain natriuretic peptide [NT-proBNP], and high sensitivity troponin level [HsTni]). Interleukin-6 was measured as a biomarker of systemic inflammation.

Statistical analysis

The primary endpoint was the association between Gal-3 expression and AKI. Results are expressed as means {plus minus} standard error of the mean (SEM). A non-parametric Mann-Whitney U test was performed unless otherwise stated. Level of Galectin-3 at discharge was compared using Kruskal-Wallis test in the clinical cohort. Univariable and multivariable analysis and using propensity score matching assessed the association between AKI and gal-3 at ICU discharge in the clinical cohort. Variables included in the multivariable analysis were age, hypertension, chronic kidney disease, atrial fibrillation, liver disease, chronic heart failure, dyslipidemia, vascular disease, cancer, body mass index, heart rate, chronic obstructive pulmonary disease, Charlson score, simplified organ failure assessment (SOFA) score, simplified acute physiology score (SAPS) 2, inotrope use, estimated glomerular filtration rate (eGFR, using the Modified and Diet Renal Disease formula), septic shock, use of red blood cell transfusion, length of stay in the ICU, gender, and arterial blood pressure. Propensity score matching considers the probability that a patient with specific baseline characteristics had an AKI and then allows the comparison of gal-3 levels in patients with or without AKI but with similar characteristics. Propensity score matching considers the probability that a patient with specific baseline characteristics had an AKI and then allows the comparison of Gal-3 levels in patients with or without AKI but with similar characteristics. Propensity score model included age, hypertension, chronic kidney disease, atrial fibrillation, liver disease, chronic heart failure, dyslipidemia, vascular disease, cancer, body mass index, heart rate, chronic obstructive pulmonary disease, Charlson score, simplified organ failure assessment (SOFA) score, simplified acute physiology score (SAPS) 2, inotrope use, renal replacement therapy, estimated glomerular filtration rate (eGFR, using the Modified and Diet Renal Disease formula), septic shock at

admission, use of red blood cell transfusion during ICU stay, length of stay in the ICU, gender , and arterial blood pressure. Matching was performed according to the nearest neighbour approach within a calliper width of 0.2(18). Imbalance between patients with and without AKI before and after PS matching was assessed using a standardized difference, considering less than 10% acceptable to define the study patients' characteristics balanced with respect to the previously described features. All statistical analyses were performed using R statistical software version 3.1.1 or above (The "R" Foundation for Statistical Computing, Vienna, Austria). A p-value<0.05 was considered statistically significant.

Results:

AKI induces gal-3 expression, cardiac injury and systemic inflammation

After renal ischemia/reperfusion in WT mice, a transient increase in creatinine (Cr) and blood urea nitrogen (BUN) levels was observed 24h post-reperfusion (**Figure 1A**). Cr and BUN returned to baseline within 48h.

AKI induced an increase in kidney gal-3 expression, mainly in tubular cells and monocytes at 24h (**Figure 1B-1D**). The increased expression of Gal3 in monocytes was also confirmed in isolated renal-macrophages (**Online Figure 1**). This increase was followed by an increase in cardiac gal-3 expression at 48h (**Figure 2A**), which was confirmed at the protein level (**Figure 2B**). Cardiac tissue infiltration by inflammatory cells and systemic inflammation assessed with plasma cytokines and adhesion molecule levels measurements, were observed during the first 72h after IR. Plasma levels of gal-3 increased, with a peak at 72h, and remained elevated until day 28 (**Figure 2D**). *CD68* mRNA expression increased in heart at 48h (**Figure 2E**). Furthermore, hearts showed CD68⁺ cells (infiltrating macrophages, **Figure 2F**) and increased

MCP-1 mRNA expression at 72h post versus sham mice ($1,49 \pm 1,0$ vs $4,1 \pm 1,8$, $p < 0.001$). Cytokines assays performed on plasma showed an increase in IL-1 β , IL-6, IL-10 and TNF- α levels at 6h and 48h after IR (**Figure 2G**). The increase in cytokine levels was accompanied by an early upregulation of *HIF-1 α* , *cTGF*, *ICAM-1* and a late increase of *VCAM-1* mRNA expression (**Figure 2H**). Furthermore, adhesion of monocytes on endothelial monolayers was significantly increased after stimulation with recombinant gal-3 (**Online Figure 2**). In the long-term, 28 days after AKI, cardiac inflammation and fibrosis were observed. Indeed, mRNA expression of both *CD68* and gal-3 was increased (**Figure 3A**). Furthermore, CD68⁺ and gal-3⁺ cells (**Figure 3B**) and increased collagen areas (**Figure 3C and 3D**) were observed.

While cardiac function was normal during the first 72h, IR induced a late increase in LV diastolic diameter (**Online Table 1**) and a decrease in fractional shortening (FS) (**Figure 3E**) 28 days after injury. At the anatomical level, kidney hypertrophy was observed 28 days after AKI and was not prevented by MCP treatment (**Online Table 1**). Interestingly, hind limb ischemia did not lead to cardiac dysfunction and injury or changes in gal-3 expression (**Online Figure 3**).

AKI-induced cardiac inflammation, fibrosis and dysfunction is gal-3 dependent

Inactivation of gal-3 by pharmacological inhibition (MCP treatment) and by genetic invalidation (gal-3 KO mice) blunted cardiac consequences of AKI. Gal-3 inactivation prevented gal-3 and cytokines release (**Figure 2D and 2G**) and cardiac endothelial activation (**Figure 2H**) during the 72 first hours after renal IR. At 28 days post AKI, gal-3 inactivation prevented cardiac monocyte recruitment, gal-3 increase (**Figure 3B**), cardiac fibrosis and cardiac dysfunction (**Figure 3C-3E**).

Gal-3 from bone marrow derived-cells is responsible of cardiac damage

AKI was performed in a graft mouse model of bone marrow. Chimeric mice were submitted to renal ischemia/reperfusion and sacrificed 28 days post-reperfusion (**Online Figure 4B and Online Figure 5**). No variations in anatomical data were observed (**Online Table 2**). Plasma gal-3 levels were close to zero in sham WT^{KO BM} mice. Furthermore, in response to renal IR, plasma gal-3 levels were higher in KO^{WT BM} than in WT^{KO BM} (**Figure 4A**). No variation in plasmatic IL-6 levels was observed at 28 days post-IR (**Figure 4B**). Cardiac *CD146* and *MCP-1* mRNA levels, an endothelial and inflammatory marker respectively, were lower in IR WT^{KO BM} vs KO^{WT BM} (**Figure 4C and 4D**). Cardiac *gal-3* mRNA expression did not change between sham and IR but varied according to mice genotype. However, gal-3 protein expression was slightly increased at 28 days after IR (**Figure E6**). Cardiac gal-3 mRNA expression was higher in KO^{WT BM} than in WT^{KO BM} (**Figure 4E**). Moreover, an increase in *ICAM-1* mRNA expression was observed in response to IR only in KO^{WT BM} (**Figure 4F**). Furthermore, Sirius Red staining showed a significant increase in cardiac interstitial fibrosis in response to IR in KO^{WT BM} compared to sham, blunted in WT^{KO BM} mice (**Figure 4G and 4H**). Interestingly, echocardiography revealed a decrease in fractional shortening after IR only in KO^{WT BM} (**Figure 4I**). Finally, CD68/galectin-3 co-staining in cardiac tissue showed CD68⁺/gal-3⁺ cells in KO^{WT BM} while CD68⁺ cells in WT^{KO BM} were gal-3⁻ (**Figure 4J**).

Cardiac damage after AKI is gal-3 dependent but renal function independent

Next, we assessed another model of renal injury, the unilateral ureteral obstruction (UUO), which does not affect renal function (**Online Figure 4C**). Indeed, levels of Cr and BUN remained normal after UUO (**Online Figure 7A**) while inflammation increased in the obstructed kidney. UUO+MCP mice were protected against renal inflammation. An increase in tubular

dilatation was observed in both UUO and UUO+MCP mice at 15 days as well as an increase in right kidney weight, synonym of renal hypertrophy, at 2 months (**Online Figure 7B and Online Table 3**). Plasma levels of TNF- α , IL-6, IL-1 β and IL-10 were also increased after UUO and peaked at 28 days except IL-1 β which rapidly increased after surgery, peaked at 7 days and then decreased and returned to baseline. The increase of cytokines levels was prevented by MCP treatment (**Figure 5A and Online Figure 8**). Plasma levels of gal-3 increased progressively during the 2 months of UUO (by four-fold compared to sham). In MCP treated mice, the plasmatic increase of gal-3 was blunted (**Figure 5A**). Similar signs of cardiac damage were observed compared with the renal IR model. An early (day 3) and transient cardiac increase in *ICAM-1* and late (2 months) increase in *ICAM-1*, *MCP-1* and gal-3 mRNA levels were observed in response to UUO, which were all prevented by MCP treatment (**Figure 5B**). At 28 days and 2 months, UUO induced cardiac inflammation and fibrosis as shown by CD68⁺ cells (**Figure 5C**) and by the increase of interstitial collagen areas (**Figure 5D**). In response to MCP treatment, fibrosis was prevented (**Figure 5C and 5D**). Importantly, UUO induced a left ventricular dilatation and a decrease in fractional shortening, prevented by MCP (**Figure 5E and 5F**).

AKI is associated with gal-3 expression and cardiac injury at ICU discharge in the clinical setting.

In the clinical cohort, six hundred and forty-five (58%) patients developed AKI during ICU stay and were discharged alive (**Online Figure 4**), including 252 subclinical AKI and 134, 65 and 194 patients with AKI KDIGO stage 1, 2 and 3 respectively. Plasma level of gal-3 showed a stepwise increase with severity of AKI (from sub-clinical to stage 3) (**Figure 6**). Plasma level of gal-3 was associated with AKI in univariable analysis (mean difference with 95% CI: 8.60

ng/mL [7.04, 10.15], $p < 0.001$) and remained significantly associated after adjustment for confounding factors in multivariable analysis (mean difference with 95% CI: 5.13 ng/mL [2.92, 7.35], $p < 0.001$). AKI was associated with increased biomarkers of cardiac injury (gal-3, sST-2 and hs-TnI), increased cardiac stress (NT-proBNP) and systemic inflammation (IL-6) at ICU discharge, even in patients who recovered their renal function (**Online Table 5**).

Discussion:

In this study, we explored the impact of AKI on remote cardiac injury. Results show that renal IR promotes the development of cardiac injury and fibrosis in part through the activation of the gal-3 pathway. Gal-3 originated from bone marrow derived cells including macrophages. Furthermore, by using the UUO model of renal disease we show that cardiac injury occurs after renal damage even if renal function is not affected. Altogether, our data indicate that the activation of the gal-3 pathway represents one of the causal links between AKI and cardiac injury.

Our findings provide important insights into cardiorenal syndrome type 3 or acute reno-cardiac syndrome (AKI leading to cardiac injury) pathophysiology(19). We hypothesized that AKI triggers the secretion of gal-3, which promotes the development of cardiac injury by generating fibrosis.

Occurrence of AKI was associated with both short and long-term risk of mortality in different settings(20). There is furthermore increasing evidence that AKI is associated with a risk of cardiovascular events(21)(22). Most recently, Go et al. explored the association between AKI and the risk of cardiovascular events during the year following hospital discharge. Using a large database with propensity score matching, they observed that AKI was a risk factor for heart failure during the year following discharge(23). Such cardiovascular consequences of AKI may

be causal (at least partially) in the poor outcomes of the injury. In this study, we explored the association between AKI, circulating expression of gal-3 and cardiac injury in critically ill patients. We observed that patients presenting AKI during ICU stay and surviving had higher plasma gal-3 levels at discharge, along with high plasma levels of biomarkers of cardiac injury and failure. While renal function does impact the level of natriuretic peptides and troponin, glomerular filtration rate doesn't appear to be the major determinant to these cardiac biomarkers level (24). Recent data show similar diurnal variation in patients with and without chronic kidney disease, suggesting that decreased clearance is not the primary mechanism for elevated hs-cTn levels in the patients with renal failure (25). Importantly, elevated levels of natriuretic peptides and troponin were associated in many studies with cardiovascular events and outcome(26)(27). Therefore, these biomarkers are still considered as valid biomarkers of cardiovascular events in patients with renal failure. Finally, patients with AKI but discharge with eGFR>60 ml.min.1.73m² showed elevated plasma biomarkers of cardiac damage compared to patients without AKI (**Online Table 5**).

We used preclinical models to explore the pathophysiological roles of gal-3 after AKI. In our models, acute cardiac response to renal injury was characterized by an early increase in plasmatic cytokine levels and cardiac injury. The acute cardiac response was followed by a late cardiac response that was characterized by systolic dysfunction and cardiac fibrosis. The early and late cardiac responses were gal-3-dependent because they were prevented by MCP, a gal-3 inhibitor, and in gal-3 KO mice. Of note, the role of gal-3 in remote cardiac injury was specifically linked to renal injury, as hind limb ischemia-reperfusion didn't induce any cardiac damage. Furthermore, gal-3 remained elevated both in the plasma and heart in the preclinical models despite complete restoration of renal function.

In short-term animal experiments, AKI was already shown to promote cellular apoptosis in the heart, followed by cardiac hypertrophy and fibrosis. Burchill et al. observed cardiac hypertrophy and fibrosis 10 days after renal IR(28). Increased levels of immunoreactive *TNF- α* , *IL-1* and *ICAM-1* mRNA were also reported in the heart within 48 hours after renal IR(29). Furthermore, cardiomyocyte apoptosis was also observed and associated with cardiac dysfunction evaluated by echocardiography at 72h(30). In another study, the macrophage chemokine osteopontin was increased along with macrophage infiltration in the heart after 24h of renal IR(31). In addition, increased *TNF- α* , *IL-6*, and *IL-1 β* plasma concentrations have been observed 3h after renal IR(32). In our model, cytokine assays showed increased plasma cytokine levels in response to AKI during the acute stage (6h-48h). This increase was prevented in MCP-treated and gal-3 KO mice. Martinez-Martinez et al. showed that experimental hyperaldosteronism leads to cardiac fibrosis in a gal-3-dependent pathway that is independent of blood pressure (8). The results from our study show that AKI promotes gal-3-dependent cardiac injury and inflammation, fibrosis and systolic dysfunction.

Recently, macrophages were identified as key players in the development of heart failure(33). Indeed, as we observed in our model, infiltration of macrophages is facilitated by the activation of the endothelium (which over-expresses on its cell surface adhesion proteins ICAM-1, VCAM-1) favoring inflammatory cells transfer from the vascular compartment to the tissue. The role of gal-3 as a chemokine was previously shown by Sano and al(34), and in addition to that, we showed that gal-3 promotes monocyte adhesion (**Online Figure 2**). Consequently gal-3 may have both chemoattractive and pro-adhesive effects locally in the damaged tissues. Gal-3 was already known for inducing fibrosis via the synthesis of TGF- β (35), but also by activating fibroblasts and collagen synthesis(36). The pro-fibrotic effect of gal-3 was observed separately

both in the kidney and the heart making this lectin a serious promoter of the type 3 cardiorenal syndrome. Modified Citrus Pectin (MCP) is a complex water-soluble indigestible polysaccharide rich in β -galactose. MCP's carbohydrate chains are rich in galactose and which are recognized by Gal-3 carbohydrate recognition domains (CRD). MCP's recognized mode of action is Gal-3 activity inhibition via CRD. There are currently no data showing anti or pro-cytokinic action of MCP via another pathway. However we can not confirm the specificity of the CRD pathway mechanism of action of MCP.

AKI can induce systemic sympathetic nervous system and renin-angiotensin-aldosterone system activation(37). Interestingly, even though increase of systemic arterial pressure is not sustained, increased vascular reactivity to angiotensin-II was reported(38). Our group and others have shown that activation of the renin –angiotensin-aldosterone system can promote cardiac and renal injuries(10)(11). Gal-3 participates in the mechanisms of aldosterone-mediated myocardial damage. It is therefore also possible that activation of the renin-angiotensin-aldosterone system promotes endothelial injury in remote organs after AKI. These vascular consequences should be investigated in the future.

Finally, cardiac fibrosis has been extensively recognized as a key player in the development of heart failure. Of note, in our models, cardiac expression of gal-3 was decreased in mice receiving MCP compared to controls and was associated with lower macrophage infiltration reflecting a possible positive feedback of gal-3 on macrophages recruitment. Thus, decrease in cardiac gal-3 expression may arise from direct synthesis inhibition as well as decrease of macrophages recruitment.

We explored the source of gal-3 in our models. Gal-3 can be expressed in different cell types including tubular and immune cells(39). Indeed, in our model of renal IR, damaged tubules

expressed gal-3 and may also be the source of proinflammatory cytokine expression. In this study, the question of the source of cardiac gal-3 after AKI was mainly explored using bone marrow transplantation. We showed that renal IR led to an increase in gal-3 expression and cardiac fibrosis and dysfunction in WT mice, but this damage were prevented in WT^{KO BM} (i.e. WT mice grafted with gal-3 KO bone-marrow). Furthermore, we observed cardiac CD68+/gal-3+ cells by immunostaining only in KO^{WT BM} while WT^{KO BM} mice did not express cardiac gal-3. This set of experiments confirms that cardiac gal-3 arises from bone-marrow-derived immune cells, including macrophages. Souza et al. showed in a mouse model of myocarditis that cardiac Gal-3 expression was high in macrophages, T cells and fibroblasts using flux cytometry and confocal microscopy. Inhibition of gal-3 by MCP or N-acetyl-D-lactosamine reduced cardiac inflammation and fibrosis, and modulated the expression of pro-inflammatory genes in the heart(40).

Gal-3 appears to be a mandatory mediator for AKI-Induced cardiac damage since specific blockage of the Gal-3 pathway prevented cardiac damage and injury. Gal-3 was shown to trigger immune cells and cytokines release. Inhibition of Gal-3 activity leads to inhibition of macrophage recruitment and activation, and therefore indirectly to a decrease in cytokine expression such as TNF-alpha, IL-1, IL-6, IL-4 or IL 8 (41, 42). The decrease in the expression of these cytokines leads to the modulation of other cytokines in the downstream inflammatory cascade. Gal-3 therefore appears up-stream of the cytokines release. It remains however to be tested whether blocking one of these downstream cytokines (IL-1, IL-6, TNF- α) would have cardio protective effects as well.

A summary scheme of our results is shown in **Figure. 7**. Kidney injury induces an increase in renal, circulating and cardiac gal-3 expression and in circulating cytokines levels. Kidney injury

leads to cardiac damage via endothelium activation, monocyte recruitment and finally development of cardiac fibrosis and dysfunction. Remote cardiac consequences of kidney injury are prevented in gal-3 KO mice, MCP-treated mice and in WT^{KO BM} mice.

In conclusion, gal-3 pathway is involved in remote cardiac damage after AKI, which may be involved in AKI-associated poor outcomes. Cardiac gal-3 originates from bone-marrow-derived cells. These findings open an area of clinical research with the aim of prevention of devastating consequences of AKI in human beings.

Perspectives:

Competency in Medical Knowledge: AKI leads to remote cardiac injuries, including cardiac inflammation, acute dysfunction, and *in fine* fibrosis. These effects may contribute to poor outcomes after AKI. Remote cardiac injury after AKI is in part mediated by a gal-3-dependant pathway, which originates mainly from bone marrow-derived immune cells.

Translational Outlook Implications: Future research should examine inhibition of gal-3 pathway after AKI to prevent adverse cardiac effects induced by AKI and improve its prognosis.

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Figure Legends:

Figure 1 - Renal ischemia/reperfusion (IR) leads to acute and transient renal dysfunction with increased gal-3 expression.

(A) Plasma assays performed in sham and IR mice showed an early and transient increase in creatinine and BUN (Blood Urea Nitrogen) levels after IR compared to sham mice ($p < 0.001$ WT IR mice vs WT sham mice). (B) Immunostaining showed increased gal-3 expression in kidney tissue, mainly in tubular cells, in response to renal IR. (C) Quantification of gal-3 immunostaining of sham, IR 24h WT, IR 24h KO gal-3 groups. After IR, gal-3 expression is increased in WT mice. (D) gal-3 co-immunostaining with α -SMA (α -smooth muscle actin, marker of smooth muscle cells/myofibroblasts), CD3 (lymphocyte marker), F4/80 (macrophage marker) and GR1 (neutrophil marker) only showed co-localization of gal-3 with F4/80 indicating that it is also expressed by infiltrated macrophages within the injured renal tissue after 24 h of IR (white arrows). N= 7-14 for WT IR groups, n= 4-7 for KO IR; n= 4 for WT and n= 4 KO gal-3 Sham. Data are presented as mean {plus minus} SEM, and comparisons of medians were made using non-parametric Mann-Whitney U test. * $p < 0.05$

Figure 2 - Cardiac inflammation and endothelial response after renal ischemia-reperfusion.

(A) Superposition of gal-3 mRNA expression kinetics in the kidney and heart showed an early increase in gal-3 in the kidney ($p < 0.001$ 24h vs. 0 h), which preceded similar observations in the heart ($p < 0.001$ 48h vs. 6 h). (B) Cardiac gal-3 protein quantification confirmed this result, and increased cardiac gal-3 levels were observed at 72 h compared to the sham group ($p = 0.016$). (C) Kidney gal-3 protein levels after IR. (D) gal-3 plasma levels began to increase at 48 h after IR compared to the sham group ($p = 0.037$), peaked at 72 h ($p = 0.017$ for WT IR mice vs WT sham

mice) and remained higher for 28 days compared to the sham group and MCP-treated mice ($p < 0.001$). (E) Cardiac CD68 mRNA increased at 48 h in WT IR mice ($p = 0.017$ vs sham group) and was significantly higher than in gal-3 KO mice ($p = 0.03$). (F) CD68 immunostaining revealed positive cells at 72 h after reperfusion in IR mice compared to sham mice. Quantification confirmed increased gal-3 expression in heart. (G) Cytokine assays performed in plasma from WT (sham, IR, IR+MCP and IR+MCP d+1) and gal-3 KO mice (sham and IR) showed a significant increase in IL1- β , IL-6, IL-10 and TNF- α levels at 6 h and 48 h in WT IR mice, compared to WT IR+MCP ($p < 0.001$), KO IR ($p < 0.001$) and WT sham mice ($p < 0.001$).

For 2-A n=4 –14. For Figure 2-B and 2-C n=4 – 7 for WT and Sham group. For figure 2-E and 2-G n=4 for sham, n= 7-14 for WT IR, n= 7-12 for WT IR + MCP, n= 4-7 for KO gal-3 IR..

Data are presented as mean {plus minus} SEM and comparisons of medians were made with non-parametric Mann-Whitney U test . * $p < 0.05$

Figure 3 - At 28 days, kidney IR-induced cardiac inflammation and fibrosis were prevented in MCP-treated and gal-3 KO mice.

(A) Compared with the IR group, gal-3 KO mice exhibited blunted cardiac inflammatory responses as indicated by lower CD68 mRNA in KO gal-3 mice ($p = 0.007$). At 28 days, cardiac expression of gal-3 remained high vs sham ($p = 0.028$). Mice treated with MCP had a lower cardiac expression of gal-3 ($p = 0.008$ for WT IR vs WT IR+MCP, and $p = 0.04$ for WT IR vs WT IR+MCPd+1 comparisons) (B) Similar results were obtained after cardiac CD68 immunolabeling; CD68+ cells (in green) were present in IR mice, while gal-3 KO and MCP-treated mice showed minimum CD68+ cells. Cardiac gal-3 immunolabeling was positive in IR hearts but negative in treated mice. Quantification of gal-3 and CD68 immunostainings confirmed the above observations ($p = 0.012$ for WT IR vs WT IR+MCP and $p < 0.001$ for WT IR

vs WT IR+MCP d+1 for CD68 immunostaining comparisons; $p < 0.001$ for both comparison WT IR vs IR+MCP and WT IR vs WT IR+MCP d+1 for gal-3 immunostaining comparisons). (C) Sirius red coloration of the IR hearts treated with MCP (IR+MCP and IR+MCP d+1) and in gal-3 KO mice revealed limited cardiac fibrosis. (D) Computer-assisted cardiac fibrosis evaluation confirmed these results ($p < 0.001$ for WT sham vs WT IR and $p = 0.003$ for WT IR vs KO gal-3 IR). (E) Cardiac function assessed by the analysis of left ventricular Shortening Fraction (SF) was altered in response to IR ($p < 0.001$ baseline vs IR) and rescued in KO and treated mice ($p = 0.002$ for IR vs IR+MCP and $p < 0.001$ for IR vs KO gal-3).

For figure 3-A, 3-B, 3-C $n = 5$ for Sham group, $n = 7-10$ for WT IR, $n = 5-6$ for WT IR + MCP, $n = 5-6$ for WT IR + MCP D+1, $n = 5-8$ for KO gal-3 IR. For figure 3-E, $n = 43$ for baseline echography and $n = 7$ for other groups. Data are presented as mean {plus minus} SEM and comparisons of medians were made with non-parametric Mann-Whitney U test. * $p < 0.05$

Figure 4 - Gal-3 from bone marrow derived cells, including macrophages, is sufficient to induce cardiac fibrosis and dysfunction.

(A) Plasmatic assays from chimeric mice $WT^{KO BM}$ (WT mice grafted with gal-3 KO bone marrow) and $KO^{WT BM}$ (gal-3 KO mice grafted with WT bone marrow) showed at 28 days a difference in plasmatic gal-3 level between sham $WT^{KO BM}$ and sham $KO^{WT BM}$. Furthermore, in response to IR, gal-3 level was highly increased at 28 days post-IR in $KO^{WT BM}$ compared to sham ($p = 0.016$), whereas in $WT^{KO BM}$ gal-3 increased was lower at 28 days post-IR ($p < 0.001$). Gal-3 level was lower in $WT^{KO BM}$ compared to $KO^{WT BM}$ ($p < 0.001$). (B, C, D, E, F) Cardiac CD146, MCP-1, gal-3 and ICAM-1 mRNAs expression were analyzed. CD146 and MCP-1 mRNAs expression were higher in IR $KO^{WT BM}$ compare to IR $WT^{KO BM}$ ($p = 0.001$ for CD146, $p = 0.005$ for MCP-1). Gal-3 mRNA level did not alter in response to IR but the level in $KO^{WT BM}$

mice was six-fold increased vs WT^{KO BM} (*p<0.001 for all comparisons). (G) Sirius red coloration showed an increase in collagen fibers in response to IR in KO^{WT BM}, blunted in WT^{KO BM}. Computer-assisted cardiac fibrosis evaluation confirmed these results. (H) Echocardiography revealed a decline in fraction shortening after 28 days post-IR in KO^{WT BM} that was prevented in WT^{KO BM} (p<0.001 for IR KO^{WT BM} vs IR WT^{KO BM} at 14 days and 28 days) (I) CD68/Galectin-3 co-immunostaining performed on WT^{KO BM} and KO^{WT BM} showed CD68+(green)/gal-3+(purple) cells in KO^{WT BM}. CD68 staining appeared to be more important and grouped after AKI. No gal-3 staining was observed in WTKO BM mice despite CD68+ cells in sham and IR mice

For 48h and 28d, n= 4 for Sham KO^{WT BM}, n= 5 for Sham WT^{KO BM}, n= 5 for IR KO^{WT BM}, n= 9 for IR WT^{KO BM}. Data are presented as mean {plus minus} SEM and comparisons of medians were made with non-parametric Mann-Whitney U test. *p<0.05

Figure 5 - Unilateral ureteral obstruction leads to cytokines release, cardiac inflammation, fibrosis and dysfunction, which is prevented in MCP treated mice.

(A) Cytokine assays performed in plasma showed a progressive increase in TNF- α , IL-6, and gal-3 levels until 28 days and 2 months respectively in response to UUO compared to WT UUO+MCP (p<0.001 for comparisons at 28 days and 56 days) and WT sham mice (p<0.001 for comparisons at 28 days and 56 days). (B) In response to UUO, cardiac mRNAs increased expressions of ICAM-1, MCP-1 and gal-3 compared to sham (UUO vs sham: p<0.001 for ICAM-1, p=0.019 for MCP-1, p=0.024 for gal-3) were prevented in MCP treated mice at 2 months post-surgery (for UUO vs UUO+MCP : p=0.002 for ICAM-1, p<0.001 for MCP-1, p=0.002 for gal-3). (C) CD68/Laminin immunostaining performed on cardiac tissue showed an increase in CD68+ cells in response to UUO from 28 days and less CD68+ cells in UUO+MCP. Quantification of CD68 staining confirmed the above observations. (p=0.0047 for UUO vs sham

and $p=0.01$ for UUO vs UUO+MCP at 28 days; $p=0.035$ for UUO vs sham and $p=0.011$ for UUO vs UUO+MCP at 2 months). (D) Sirius red coloration revealed cardiac fibrosis after 2 months of UUO. MCP treatment blunted collagen accumulation and deposition. Computer-assisted cardiac fibrosis evaluation confirmed these results ($p<0.001$ for both comparisons UUO vs sham and UUO vs UUO+MCP). (E) Left ventricular Fractional shortening (FS) analysis by echocardiography after 2 months highlights a progressive decreasing in FS in response to UUO ($p=0.01$ for UUO vs sham), prevented in treated mice ($p=0.02$ for UUO vs UUO+MCP). Left ventricular end diastolic diameter (LVEDD) analysis revealed an increase in LVEDD after 2 months for UUO ($p=0.043$ UUO vs sham), reflecting left ventricular dilatation. For different time points, $n= 4$ for WT sham UUO, $n= 5-8$ for WT UUO, $n= 6-8$ for WT UUO + MCP. Data are presented as mean {plus minus} SEM and comparisons of medians were made with non-parametric Mann-Whitney U test. * $p<0.05$

Figure 6- Plasma gal-3 and AKI in critically ill patients discharged from intensive care unit.

(A) Plasma gal-3 level at hospital discharge according to AKI stage (sub-clinical, and stage 1, 2 or 3 of the KDIGO guidelines). We observed a stepwise increase of plasma gal-3 level with AKI stages. (B) Graphical representation of imbalance in patients' characteristics before and after propensity score (PS) matching between no AKI and AKI patients (Black squares represents mean standardized difference (MSD) before PS-matching and the red points MSD after PS-matching).

Figure 7 - Schematic representation of the results from initial renal injury to cardiac dysfunction, including the effects of gal-3 inhibition

Renal injury induced transient renal dysfunction only in IR model (increase in creatinin and BUN), tubular cell response and macrophage activation, which led to an increase in gal-3 expression. These features led to systemic inflammation by increasing levels of plasma cytokines (IL-1, IL-6, IL-10 and TNF-a) and gal-3, which induced cardiac endothelial activation as shown by increased in ICAM-1 (Inter Cellular Adhesion Molecule-1), VCAM- 1 (Vascular Cell Adhesion Molecule-1), MCP-1 (Monocyte Chemo attractant Protein-1) and cTGF (connective Tissue Growth Factor) mRNA levels. Endothelial activation promoted monocyte infiltration in cardiac tissue; these monocytes differentiated into activated macrophages (increased CD68, Cluster of Differentiation 68), thus expressing and secreting TGF β (Transforming Growth factor β) and gal-3. TGF β and gal-3 may participate in the activation of fibroblasts leading to Coll-1 and 3 (Collagen type 1 and 3) synthesis and subsequent cardiac fibrosis and dysfunction. Treatment with MCP (Modified Citrus Pectin), gal-3 deletion (gal-3 KO mice) and WT mice grafted with gal-3 KO BM, prevented the increase in plasma cytokines, gal-3, and cardiac gal-3, fibroblast activation and the increase in cardiac fibrosis, which therefore prevented cardiac dysfunction.

Supplementary Materials:

Online data supplements – Methods

Online data supplements – Figures and Tables:

Online Figure 1 - Schematic representation of different experimental protocols.

Online Figure 2 - Schematic representation of bone marrow graft between WT and Gal-3 KO mice.

Online Figure 3 - Gal-3 expression on isolated macrophages from renal tissue.

Online Figure 4- Monocyte adhesion is Galectin-3 dose dependent.

Online Figure 5- Hind limb ischemia did not lead to cardiac injury.

Online Figure 6 - Gal-3 protein expression in heart at 48h and 28d after IR.

Online Figure 7 - UUO leads to renal inflammation and tubular damage without renal dysfunction.

Online Figure 8- UUO induces increase of plasmatic IL-1 and IL-10 levels.

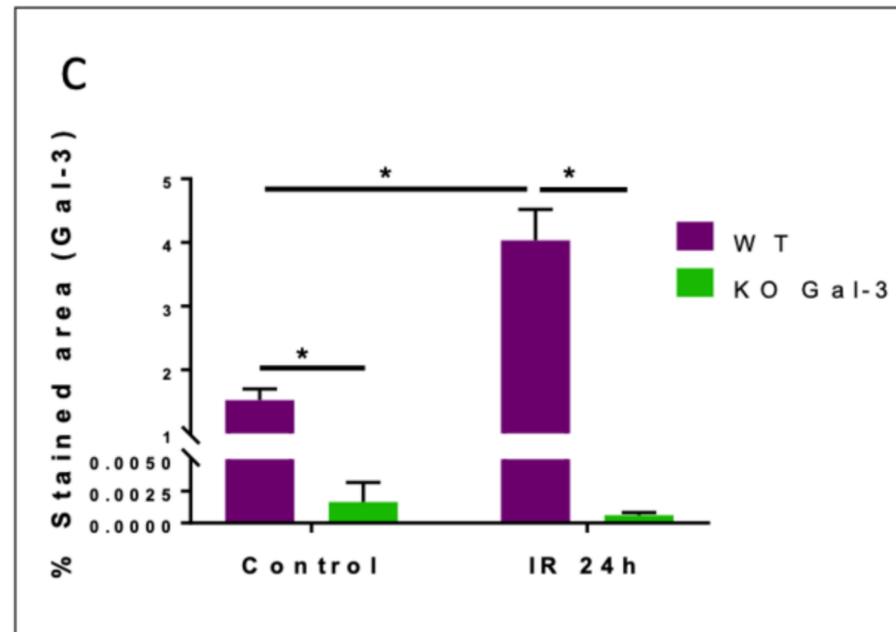
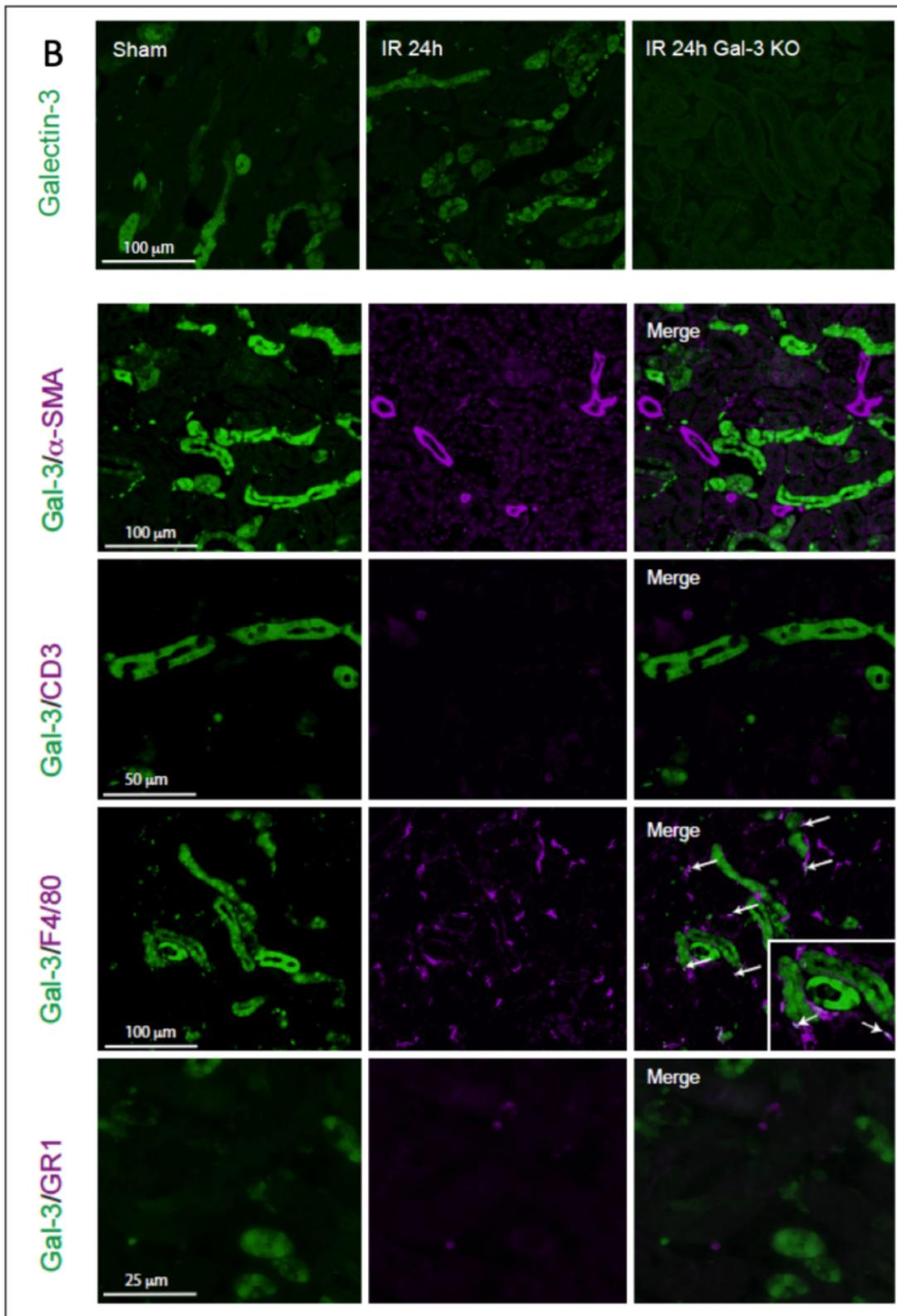
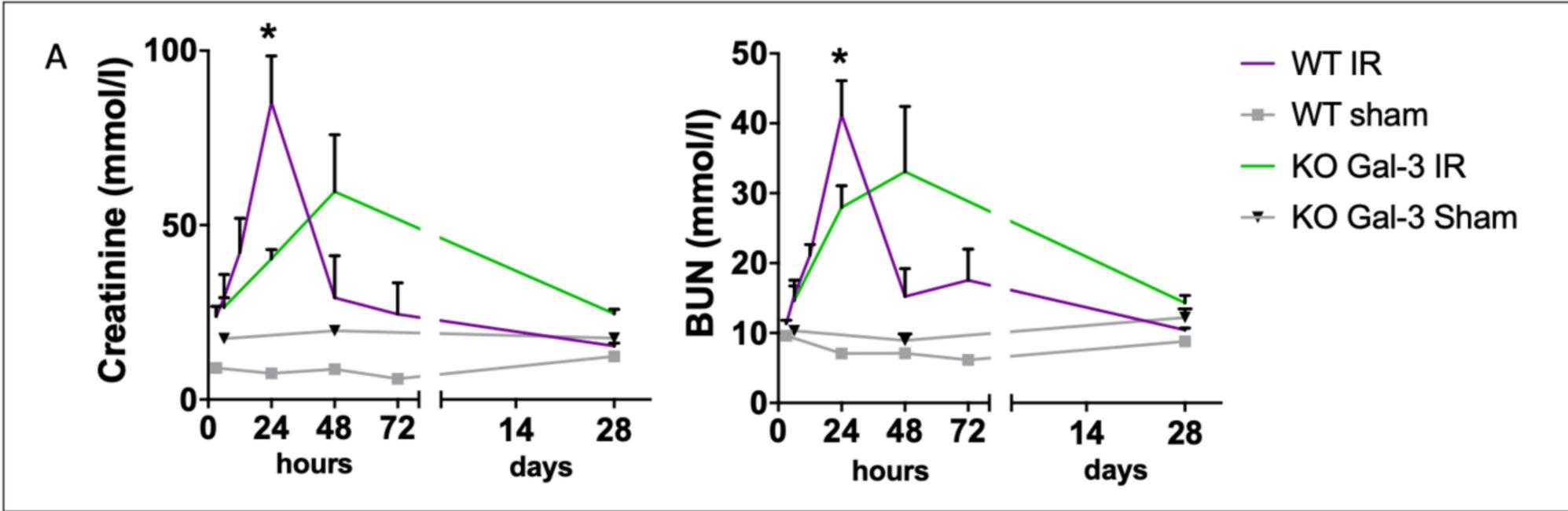
Online Table 1 - IR mice anatomical data and echographic analysis at baseline and 28 days.

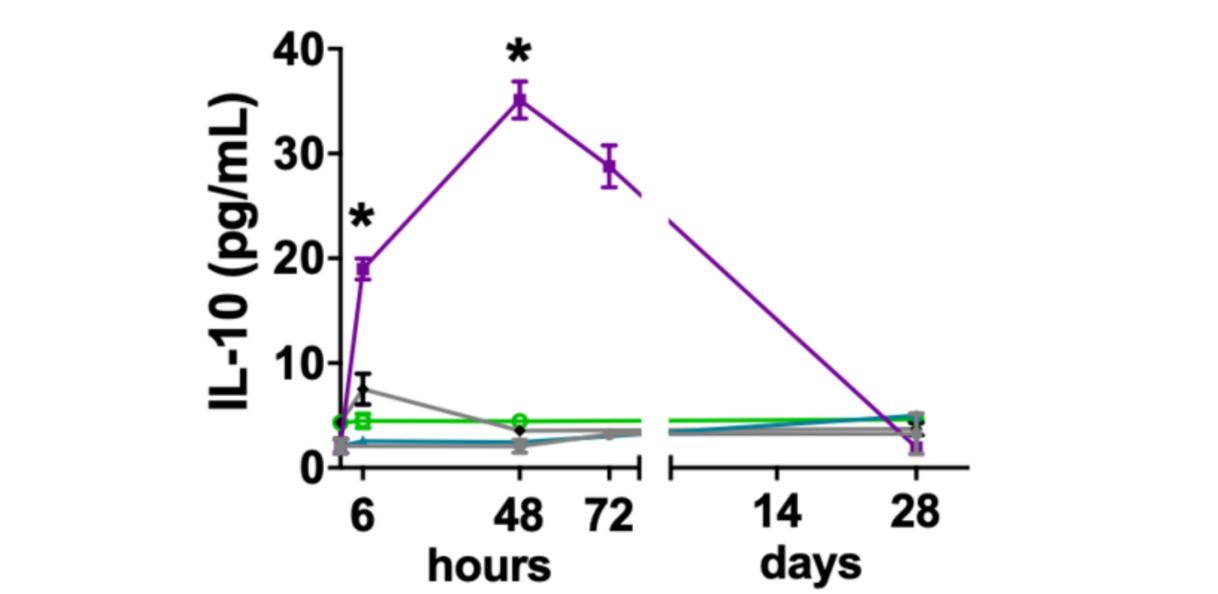
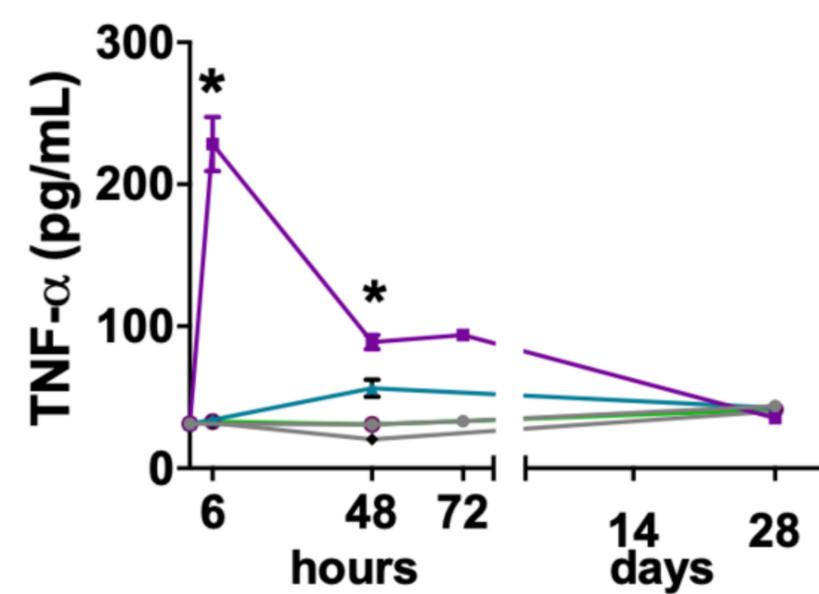
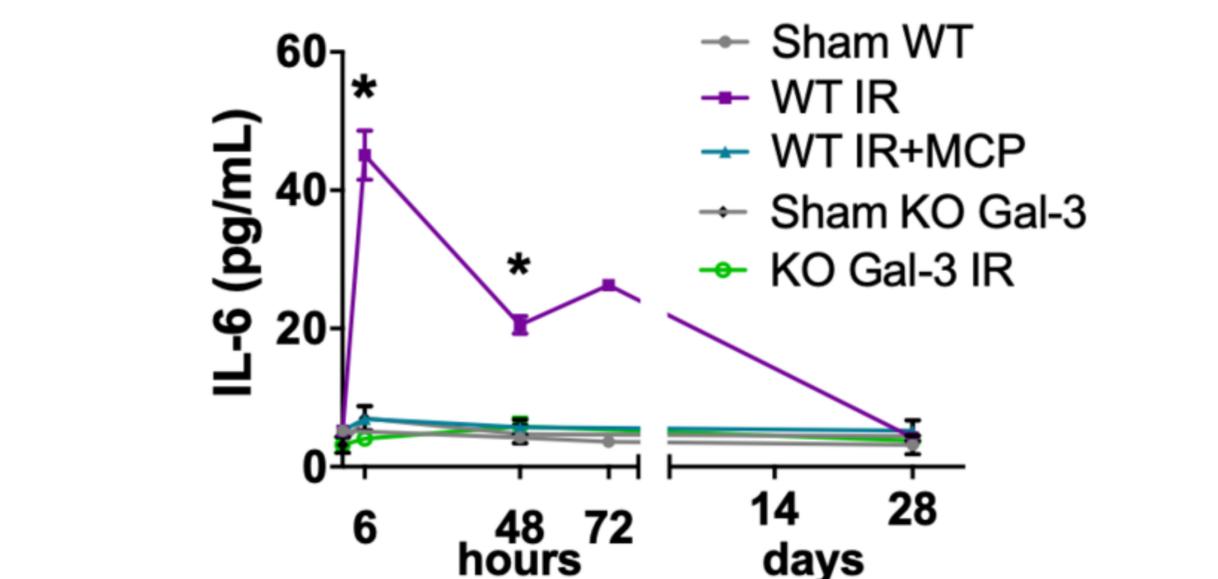
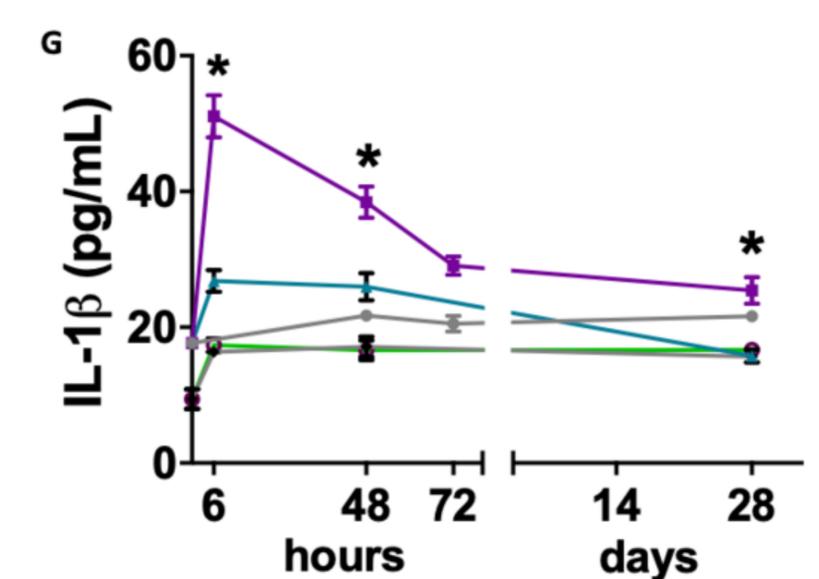
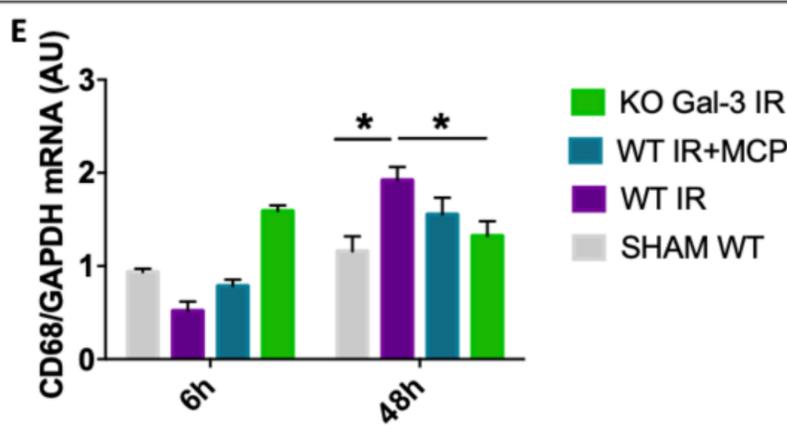
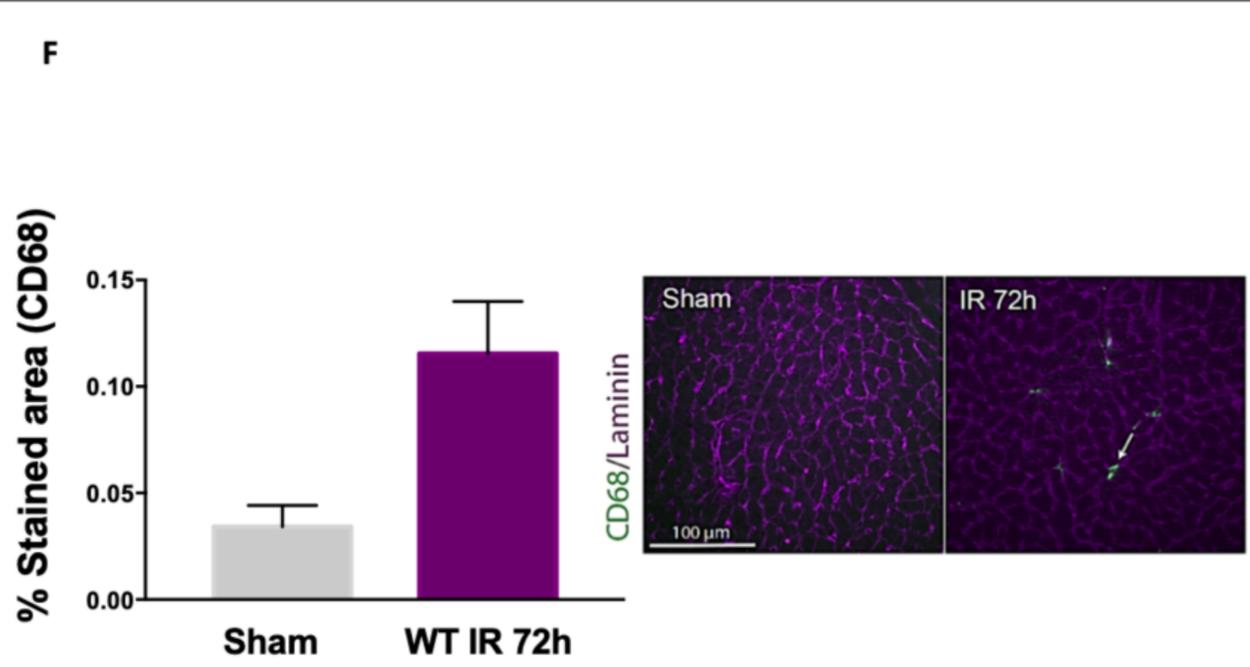
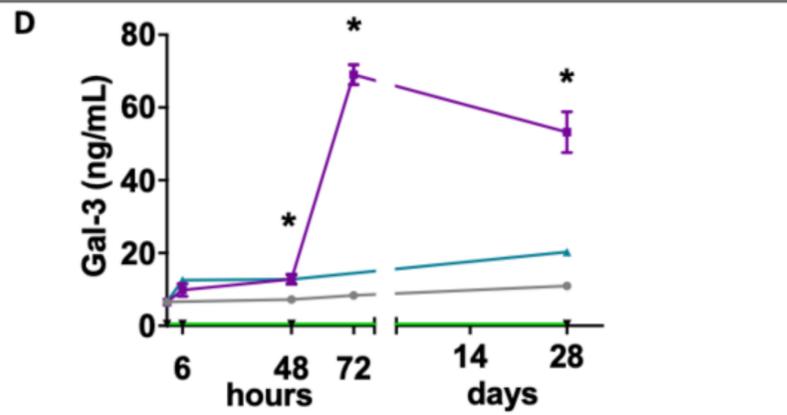
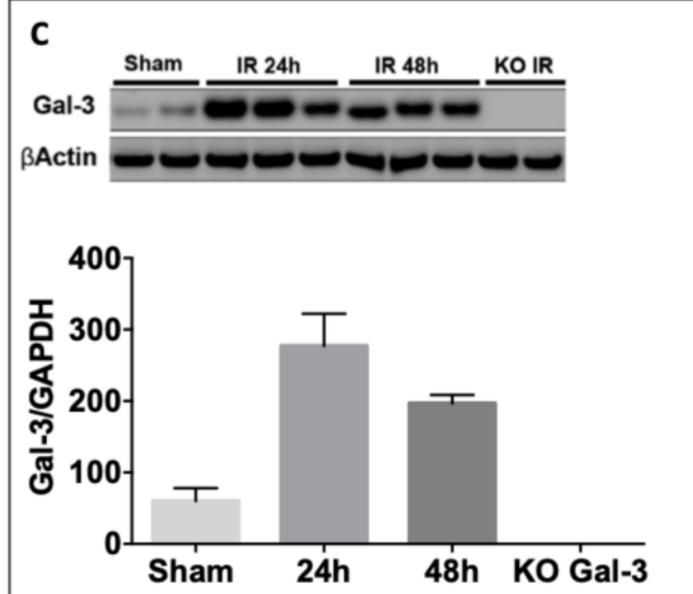
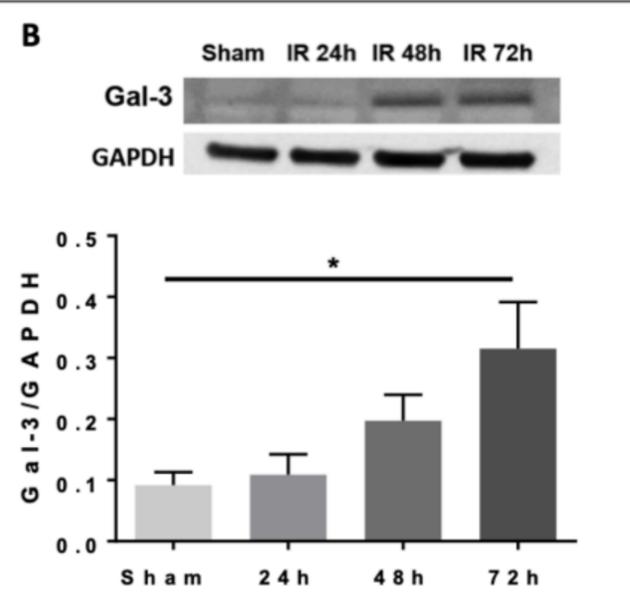
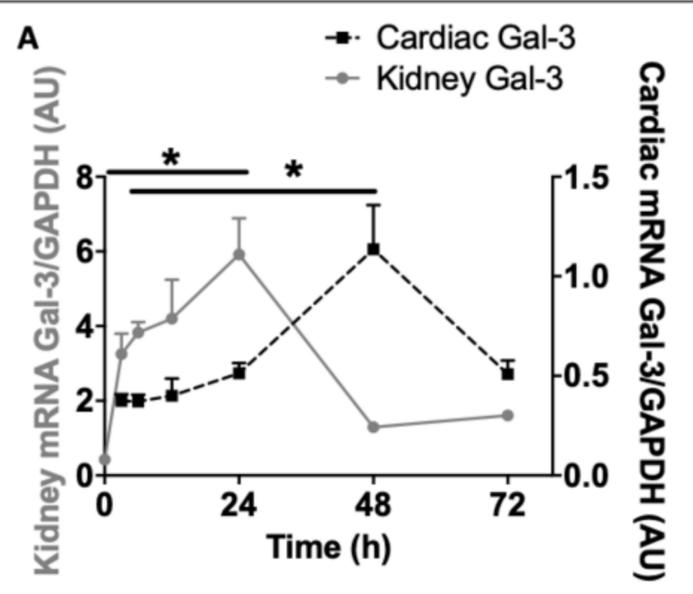
Online Table 2 - Anatomical data from bone marrow grafted mice.

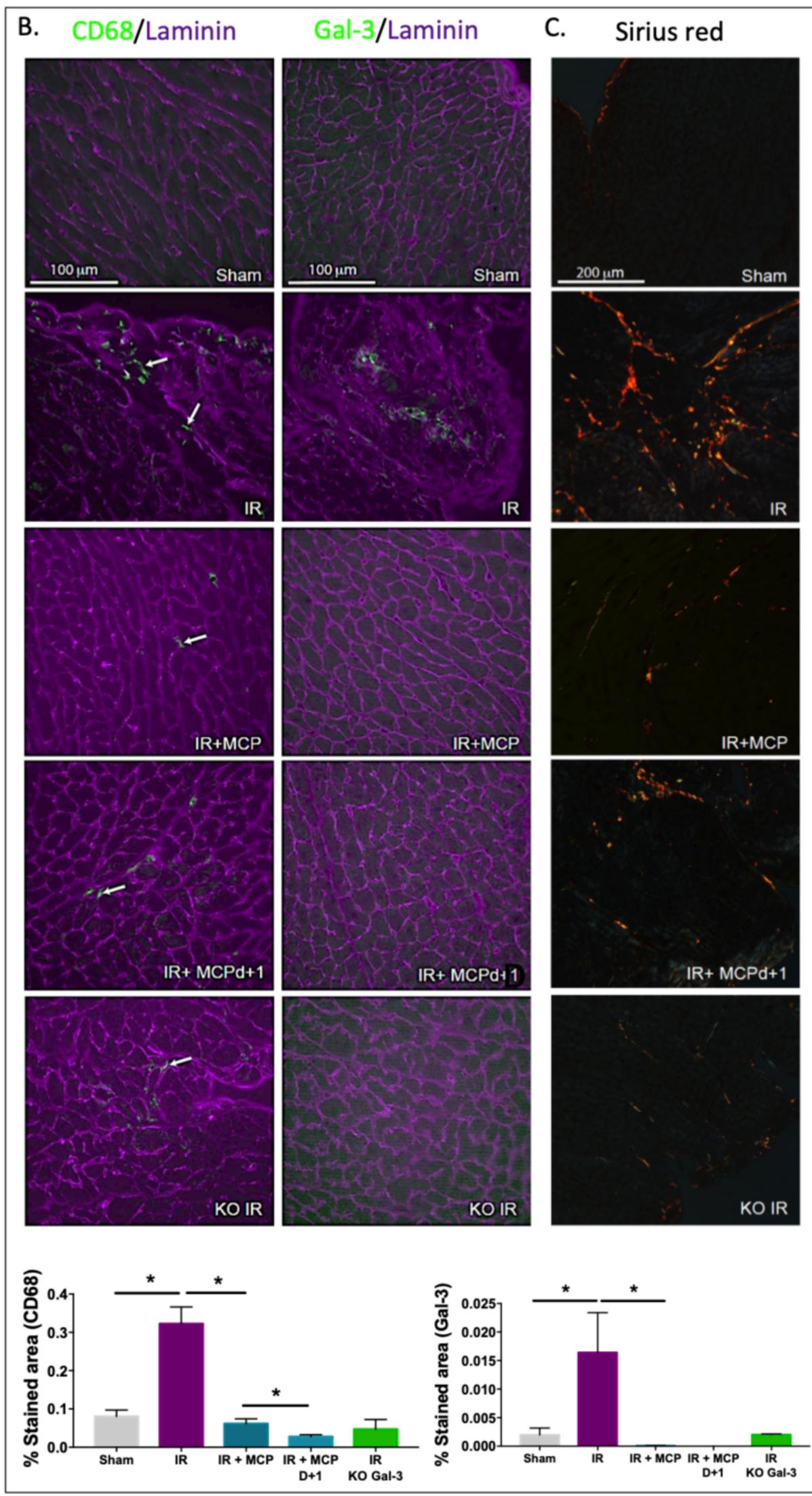
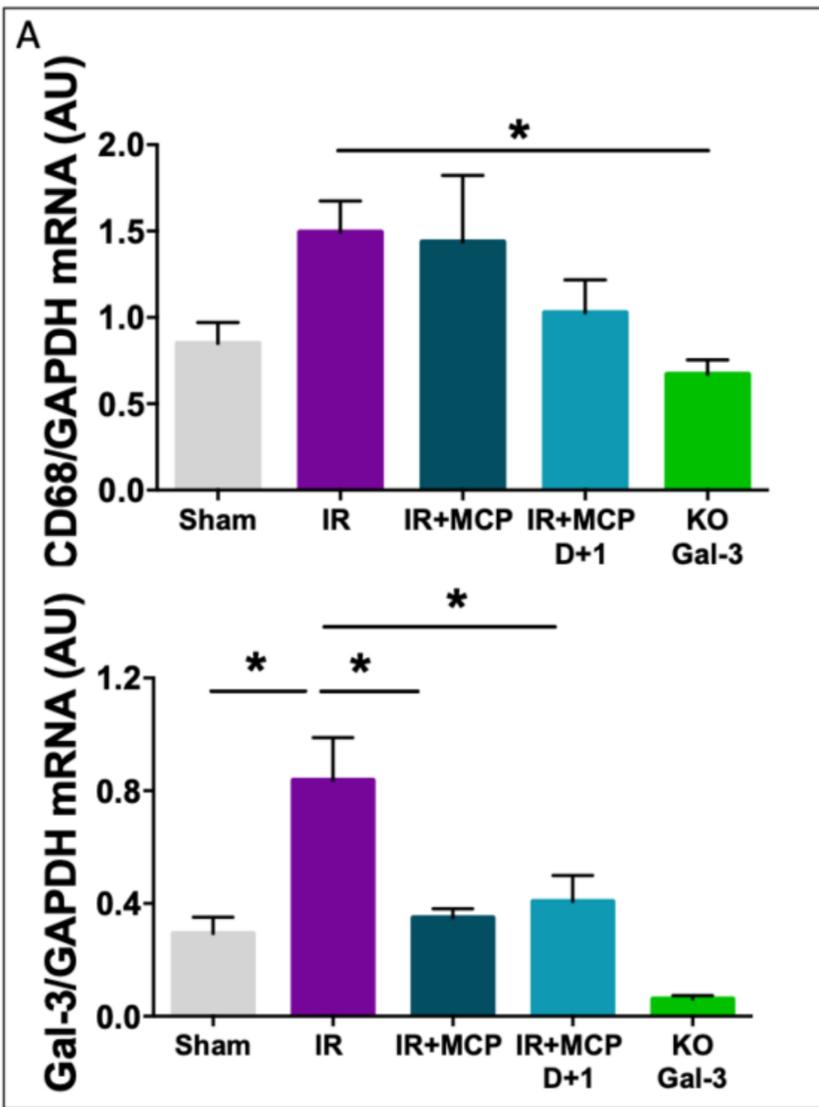
Online Table 3 - Anatomical data in mice after UUO.

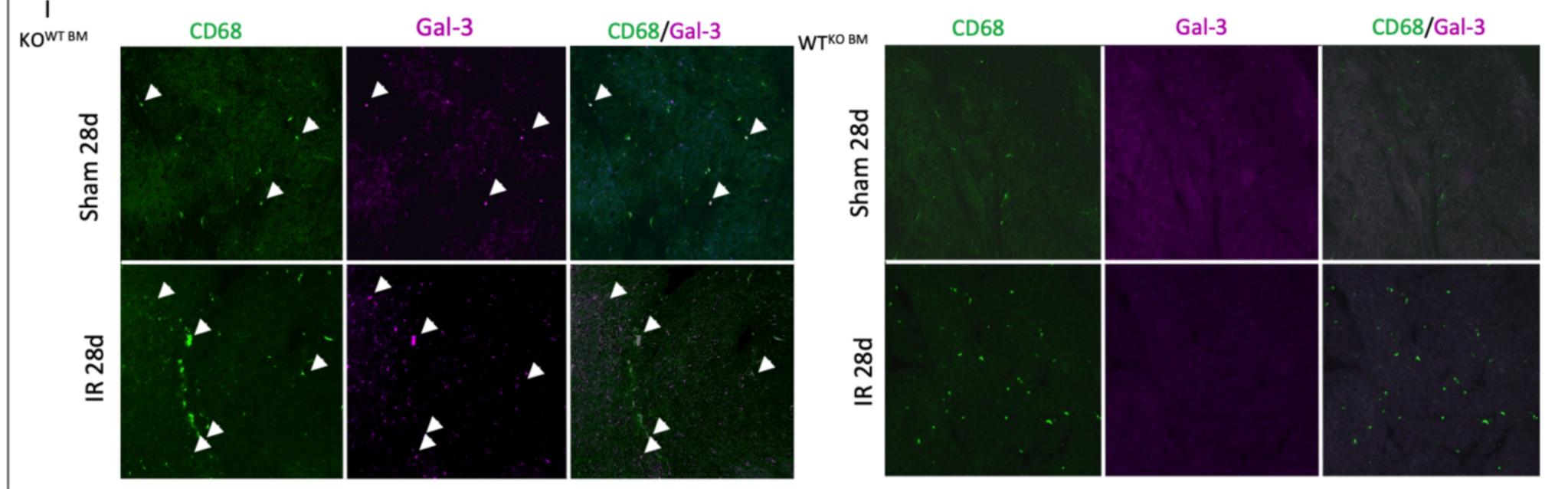
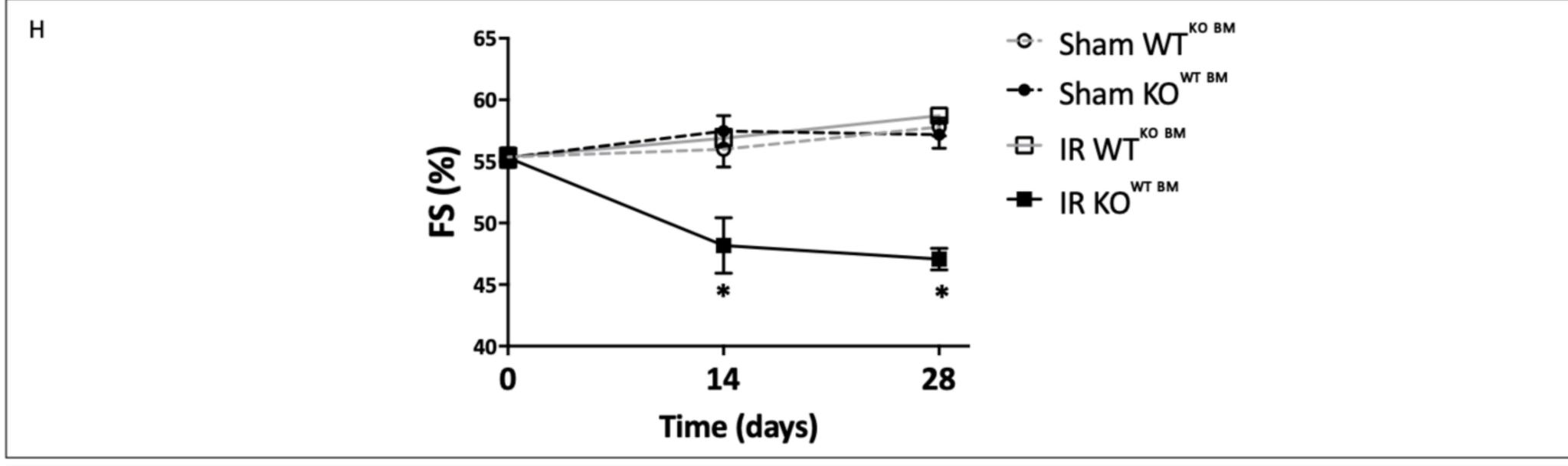
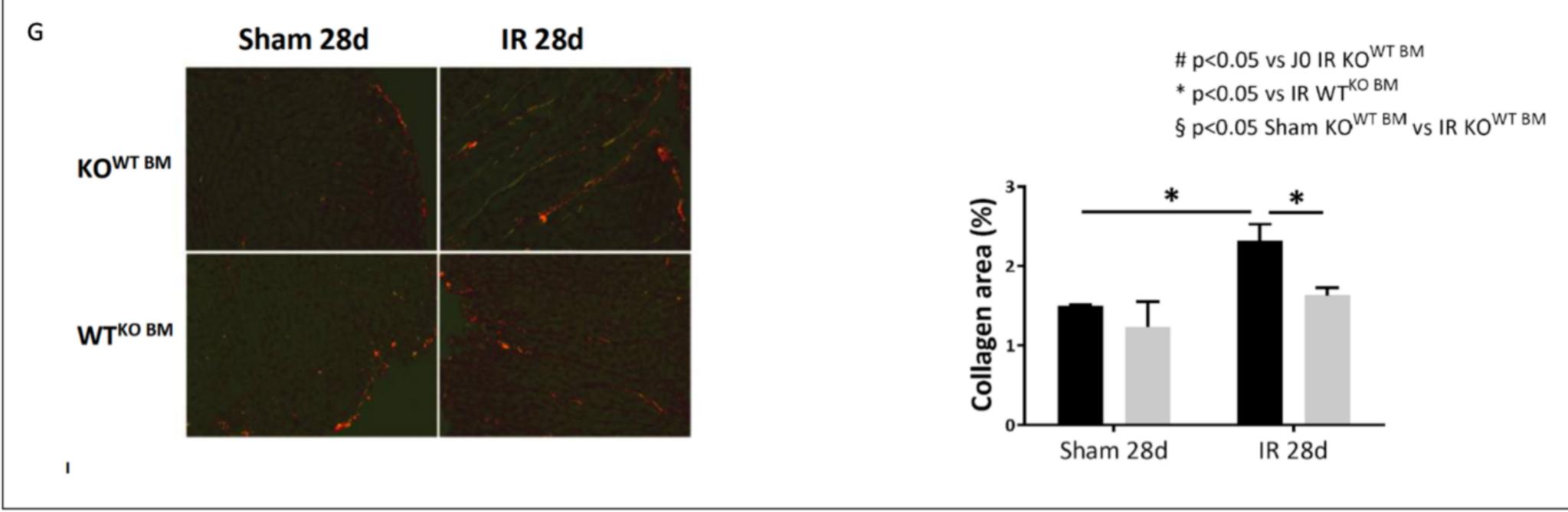
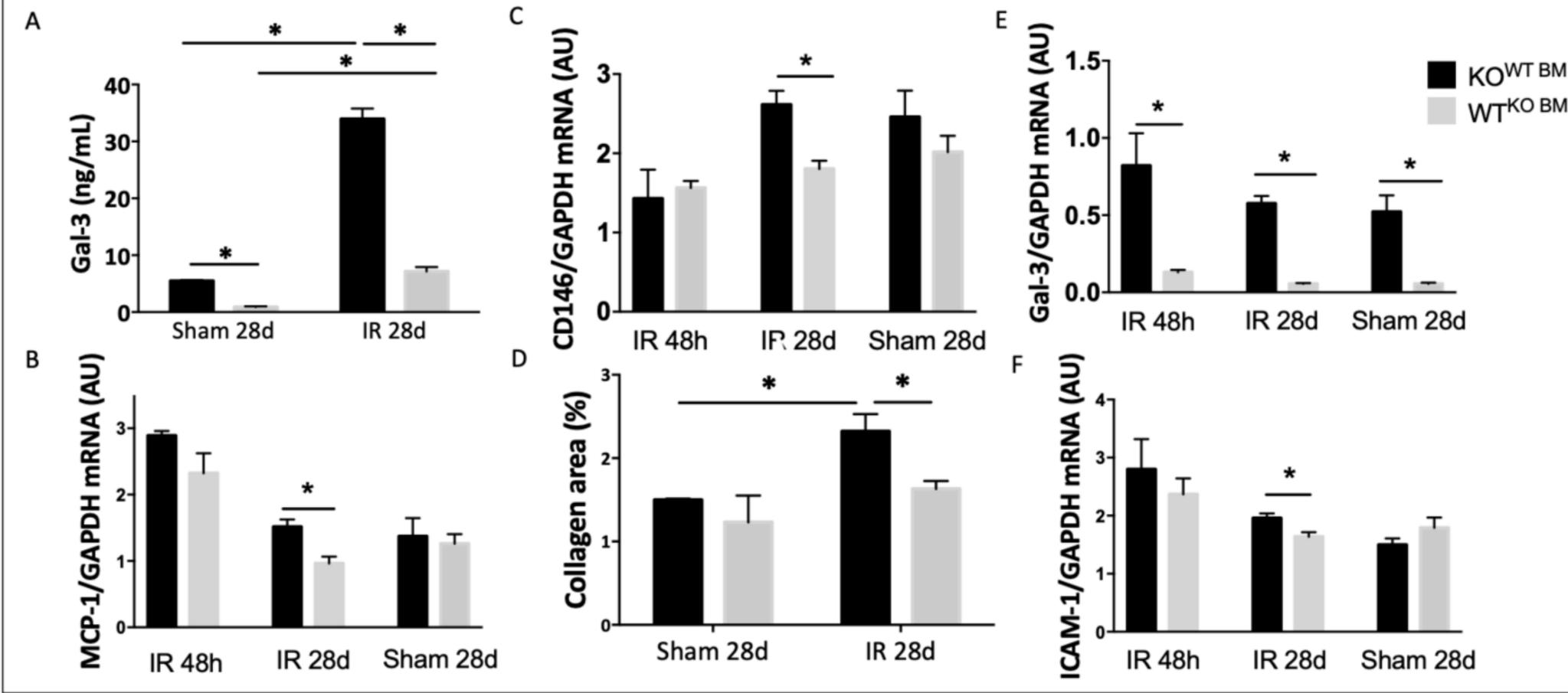
Online Table 4 - Characteristics of patients included in the clinical cohort, before and after propensity score matching according to the occurrence of acute kidney injury (AKI) at intensive care unit (ICU) admission.

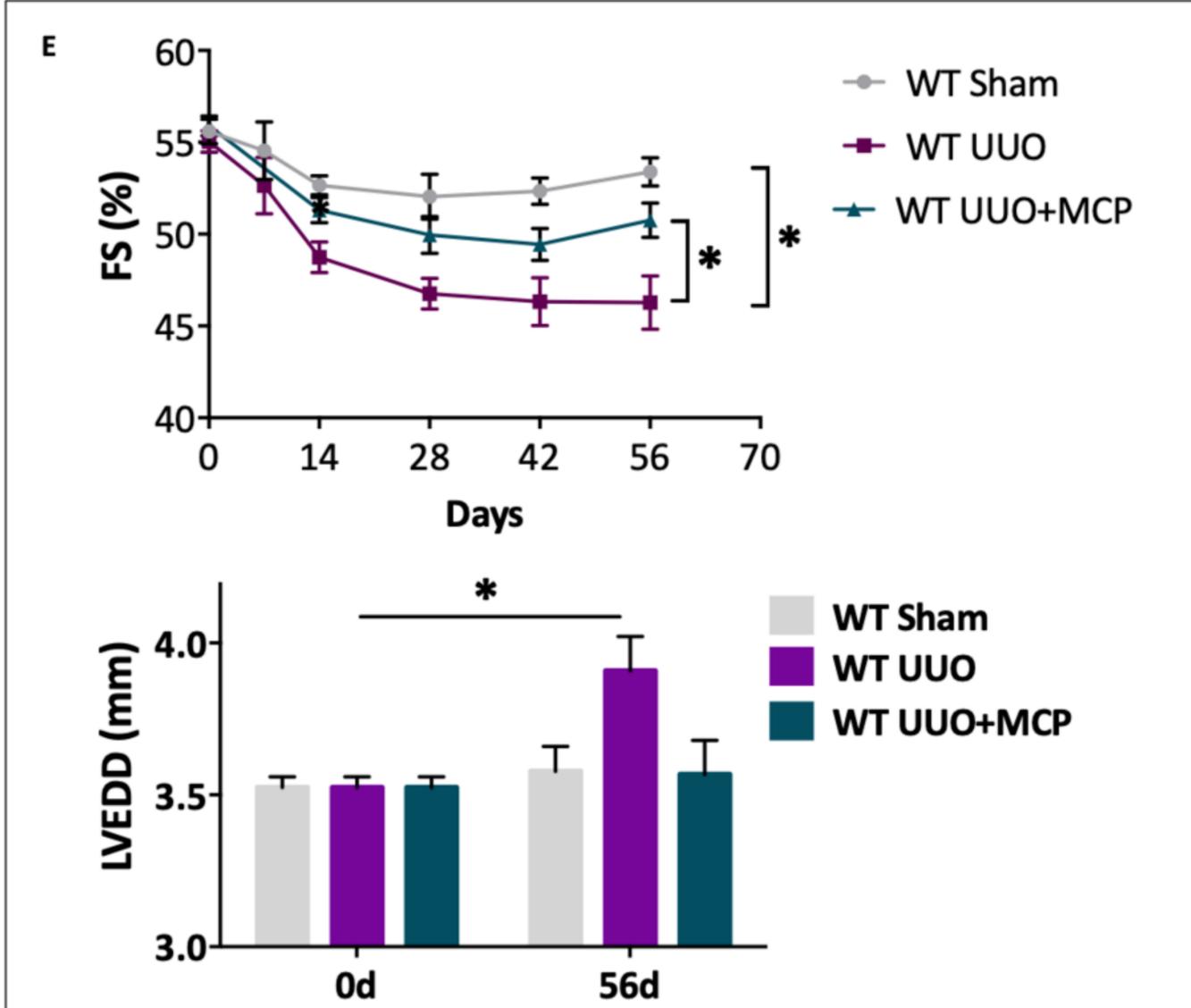
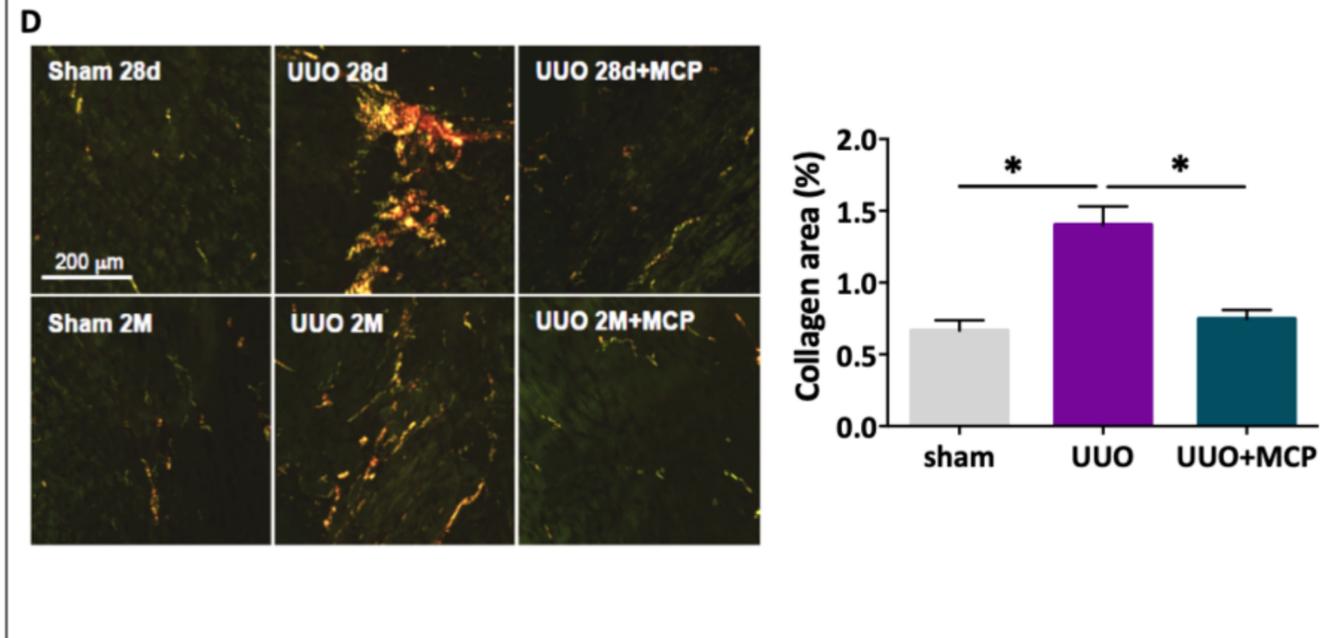
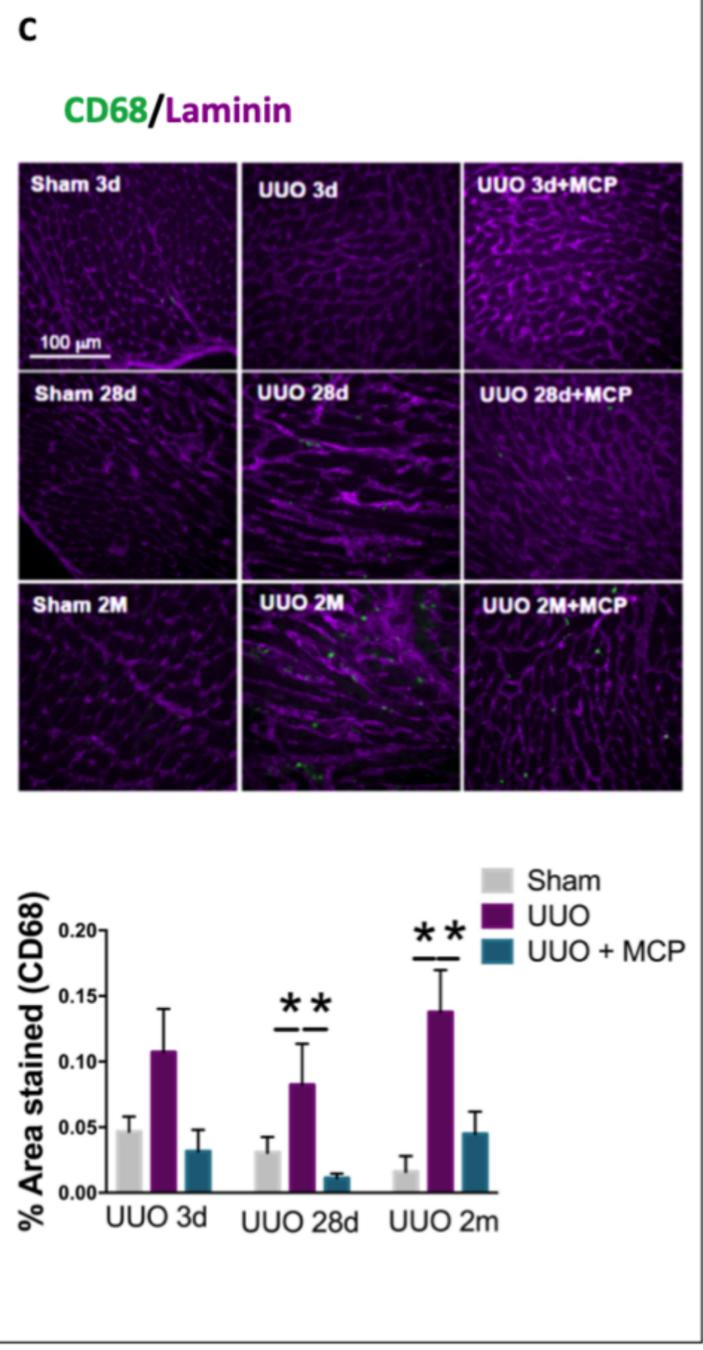
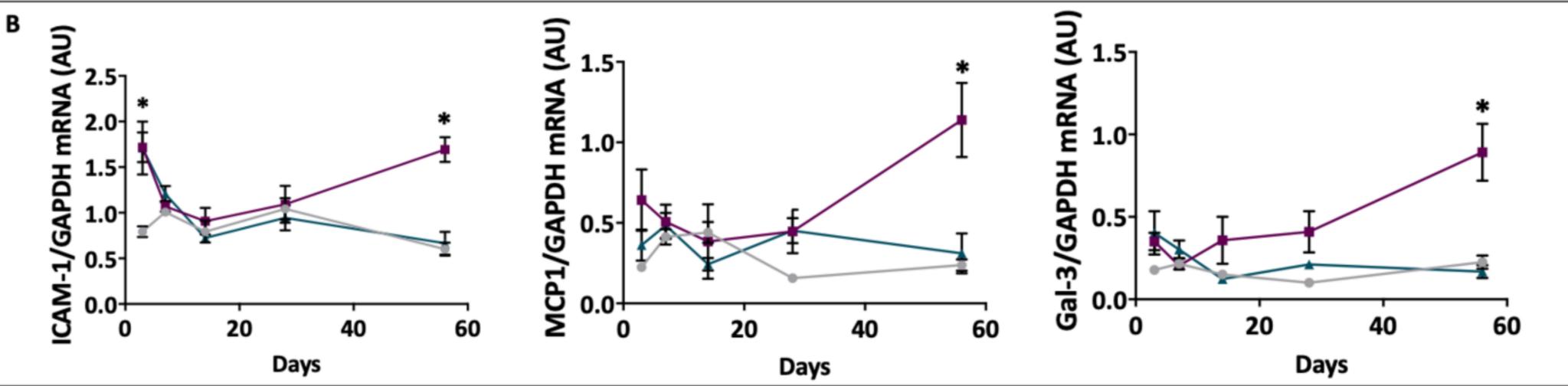
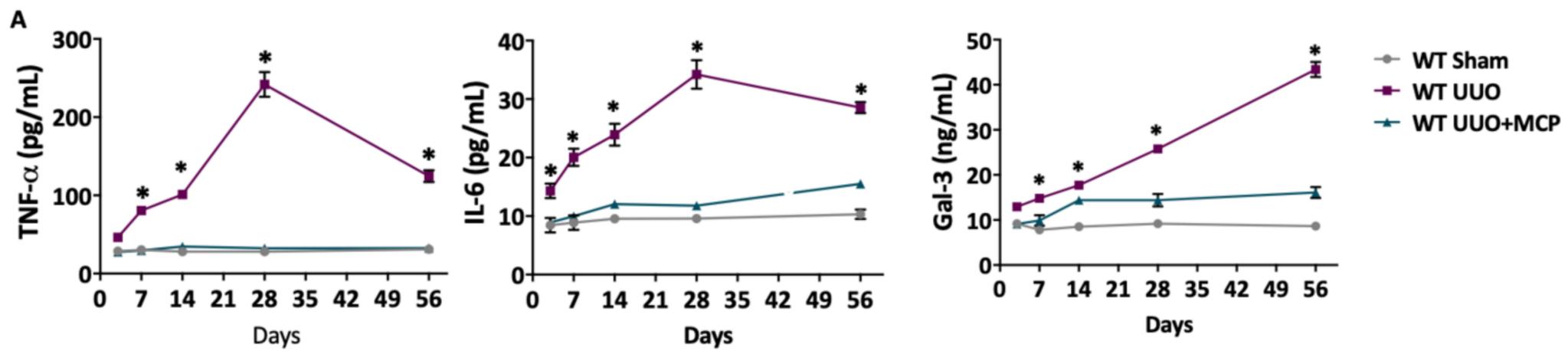
Online Table 5 - Cardiac injury, cardiac stress and Interleukin (IL)-6 biomarkers level in patients with and without AKI.

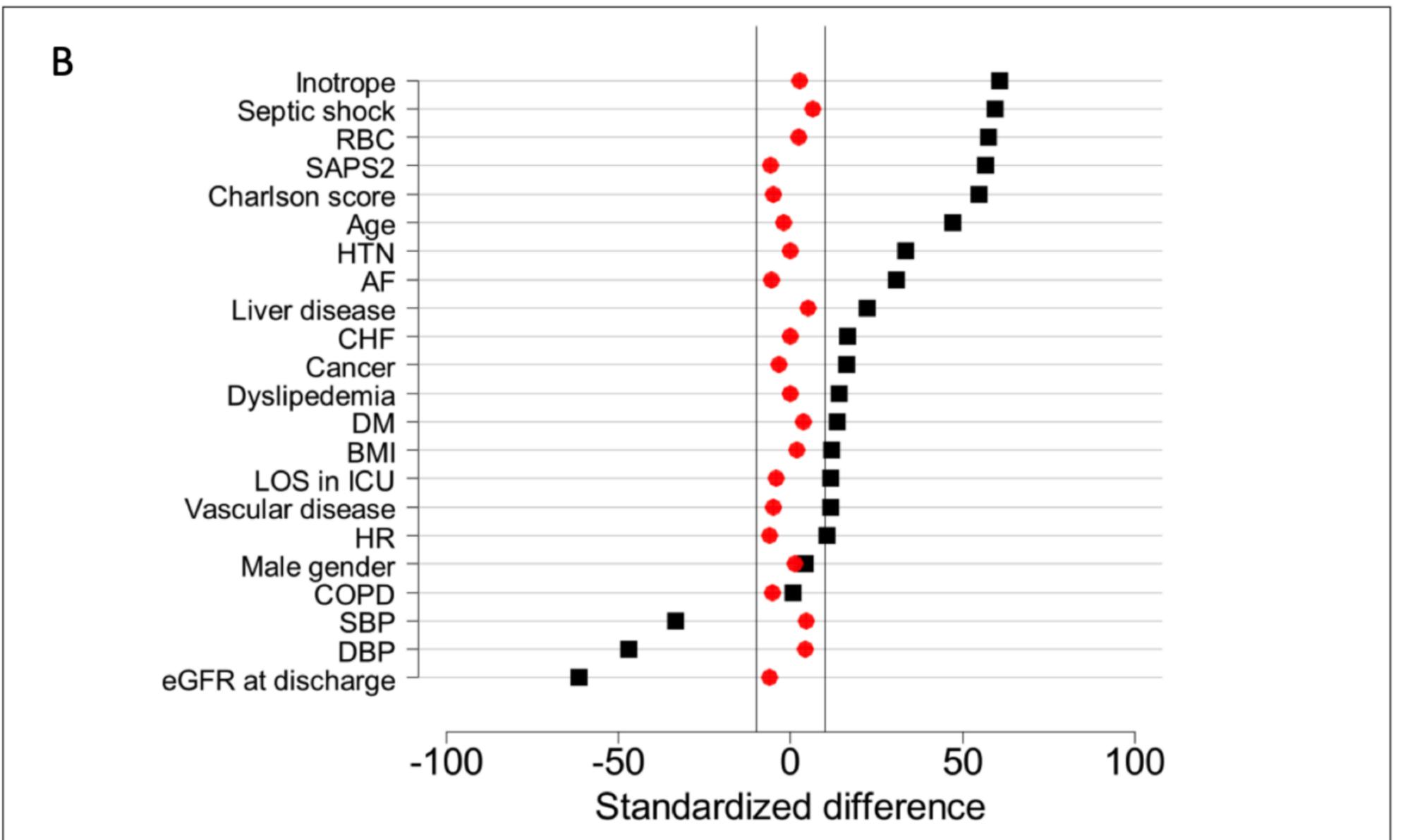
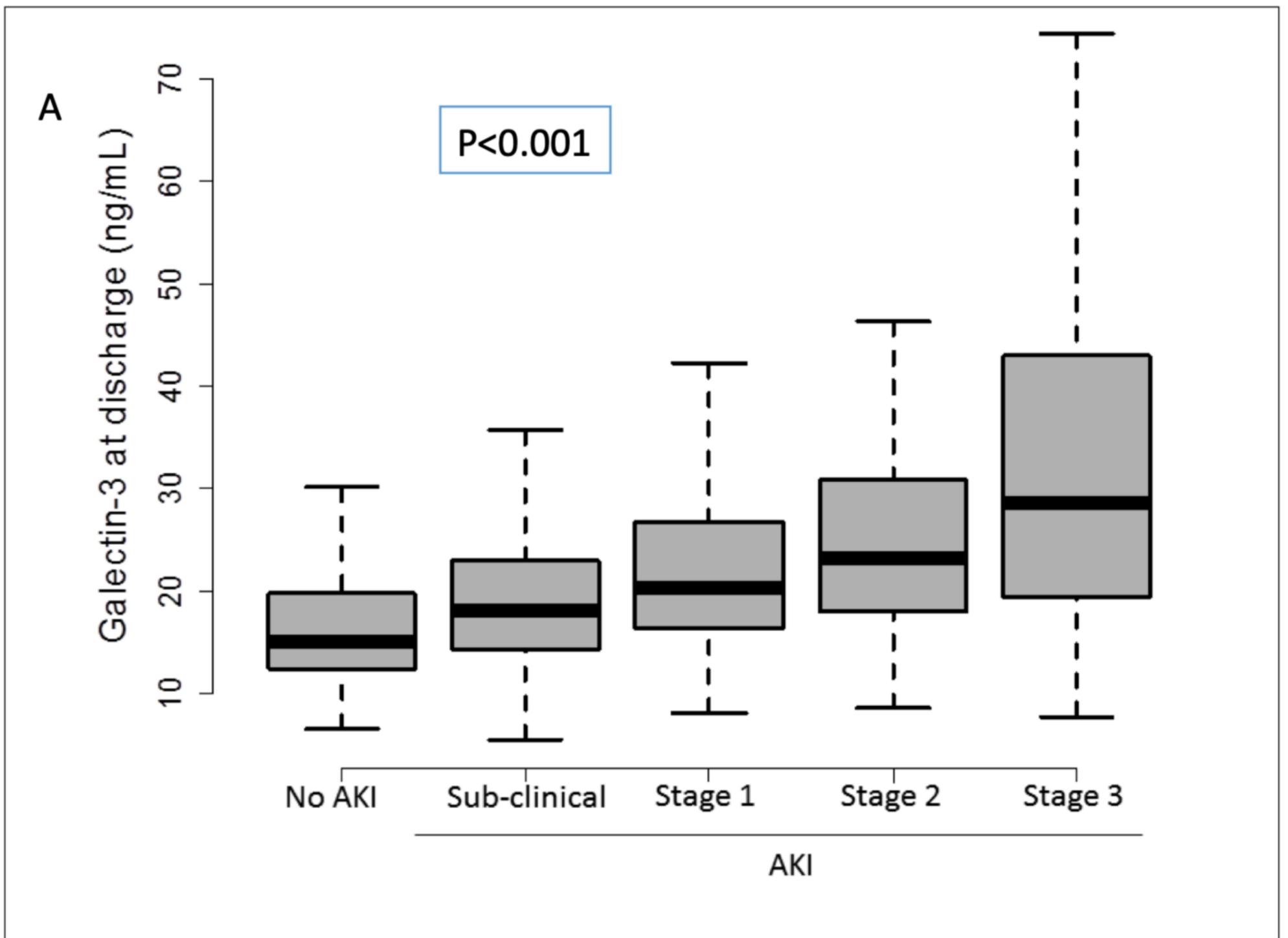












Kidney Injury

Plasma compartment

↗ Cytokines:
(IL-1/IL-6/IL-10/TNF- α)

↗ Gal-3

Endothelial activation



Bone-marrow-derived monocytes infiltration

Activated macrophages

↗ Gal-3

Activated fibroblasts

Cardiac fibrosis & dysfunction