**Urinary extracellular vesicles and micro-RNA as markers of acute kidney injury after cardiac surgery – Supplementary meterial**

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**Supplementary Methods**

**Biochemical biomarkers** Tissue metalloproteinase 2 (TIMP2) and insulin-like growth factor binding protein-7 (IGFBP7) were measured in urine using the human TIMP-2 Quantikine ELISA kit (Biotechne) and human IGFBP7 ELISA kit (abcam). Neutrophil gelatinase-associated lipocalin (NGAL) was measured in urine using Human Lipocalin-2/NGAL Quantikine ELISA Kit (BioTechne), Serum troponin was measured using High-Sensitivity Troponin I kit on Advia Centaur XP. Serum NT-proBNP was measured using NT-proBNP human ProcartaPlex™ Simplex kit (ThermoFisher) on the MAGPIX platform (Luminex).

**EV analysis** EV concentrations and size distributions were tested with NanoSight NS500 (Malvern Panalytical) in 1 ml aliquots of urine diluted 1:50 with 0.2mm filtered PBS. Flow cytometry analysis was performed in 50 µl urine. All reagents (apart from antibodies) were filtered through 0.2mm filters. Unspecific binding was blocked for 10 min with 15% BSA and 0.3 µg/ml human IgG. Antibody labelling was performed for 25 min at room temperature using 0.5 - 1 µg PE-coupled IgG against CD143 (clone 5-369, Biolegend), CD144 (clone 16B1, Life Technologies/ThermoFisher), CD105 (clone MJ7/18, Life Technologies/ThermoFisher) and FITC-coupled CD284 (clone HTA2125, ThermoFisher), CD29 (TS2/16 clone, Life Technologies/ThermoFisher) and activated CD29 (HUTS-21 clone). For control, fluorophore-coupled isotype control IgGs were used (Life Technologies/ThermoFisher). Data acquisition was performed on CytoFLEX flow cytometer (Beckman Coulter) optimized with Submicron Bead Calibration kit (Polysciences). Exosome analysis was performed in 50 µl urine. Exosomes were first bound to CD81 magnetic beads (Invitrogen, ThermoFisher) and incubated for 30 min with 15% BSA and 0.3 µg/ml human IgG to block unspecific binding. Antibody labelling was performed for 25 min at room temperature with 0.5 - 1 µg IgG against aquaporin-2 (AQP2, clone E-2, Santa Cruz Biotechnology), podocalyxin (PODXL, polyclonal, Proteintech Europe) and sodium–hydrogen antiporter 3 (NHE3, clone 14D5, Insight Biotechnology) followed by incubation with Ax594-coupled secondary antibodies (Life Technologies/ ThermoFisher). For controls, isotype control IgGs were used. Data acquisition was performed on CytoFLEX gated for CD81 beads.

**miR analysis** EV's RNA was isolated from 1ml urine with an exoRNeasy kit (Qiagen) according to the manufacturer's recommendations. Five microliters of the RNA preparation was used for sequencing library preparation using QIAseq miRNA Library kit (Qiagen). Library quality was assessed on BioAnalyzer 2100 with High Sensitivity DNA chip (Agilent). The presence of a peak of approximately 180kb indicated successful library preparation. Libraries concentrations were calculated according to recommendations in the QIAseq kit. Sequencing (75bp single reads) was performed on MiSeq (Illumina), aiming at a minimum of five million reads per sample.

The sequencing data were quality-checked with Fastqc v0.11.5, [1] aligned to miRBase 21 [2] using bowtie2 [3] as described in [4] and quantified using Cufflinks [5]. FPKM quantities were analyzed for differential expression using *limma* R-package.

Quantitative real-time PCR was carried on Qiagen's Rotor-Gene machines using TaqMan Advanced miRNA assays (ThermoFisher) for miR-10a-5p, miR-125a-5p, miR-196a-5p, miR-320a-3p, miR-93a-5p, miR-99a-3p, miR-99a-5p and miR-21-5p. let-7a-5p was used as a reference control, since its levels did not change between the groups or over time.

**Table S1 – Pre- and Postoperative characteristics in the sequenced cohort.**

(*) - Tests between groups were conducted by exact test for categorical variables and ANOVA or non-parametric Kruskal-Wallis test for continuous variables. Data are presented as n (%) for categorical variables and mean (standard deviation, STD) or median (interquartile range, IQR) for continuous variables. Abbreviations: ACE – Angiotensin-Converting Enzyme; AKI – Acute Kidney Injury; CABG – Coronary artery Bypass Grafting; CCS – Canadian Cardiovascular Society; Hct – Hematocrit; FiO2 – Fraction of Inspired Oxygen; KDIGO - The Kidney Disease Improving Global Outcomes; MODS – Multiorgan Dysfunction Syndrome; NYHA – New York Heart Association; PO2 – Partial Pressure of Oxygen; RBC – Red Blood Cells; VD – Vessel Disease.

| **n = 10** | | **No AKI n=5** | **AKI n=5** | ***P*-value*** | **Missing data (n)** |
|---|---|---|---|---|---|
| Age (years) - Median (IQR) | | 71 (7.55) | 71.2 (3.35) | 0.96 | 0 |
| Sex (female) - n (%) | | 2 (20%) | 1 (10%) | 1 | 0 |
| Ethnic (Caucasian) - n (%) | | 5 | 5 | 1 | 0 |
| Sildenafil intervention | | 2 (20%) | 1 (%) | 1 | 0 |
| Diabetes - n (%) | | 0 (0%) | 0 (0%) | NA | 0 |
| Stroke/Transient Ischaemic Attack - n (%) | | 0 (0%) | 0 (0%) | NA | 0 |
| Chronic obstructive pulmonary disease - n (%) | | 0 (0%) | 0 (0%) | NA | |
| Renal disease - n (%) | | 0 (0%) | 0 (0%) | NA | 0 |
| Myocardial infarction - n (%) | | 0 (0%) | 0 (0%) | NA | 0 |
| Pulmonary hypertension - n (%) | | 0 (0%) | 0 (0%) | NA | 0 |
| Anemia n (%) | | 3 (30%) | 1 (%) | 0.52 | |
| Surgery type | CABG - n (%) | 3 (30%) | 1 (%) | | |
| | Valve - n (%) | 0 (0%) | 1 (%) | | |
| | CABG & Valve - n (%) | 2 (20%) | 2 (%) | | |
| | other - n (%) | 0 (0%) | 1 (%) | 0.71 | 0 |
| NYHA | Class I - n (%) | 0 (0%) | 1 (%) | | |
| | Class II - n (%) | 3 (33.33%) | 3 (%) | | |
| | Class III, IV - n (%) | 1 (11.11%) | 1 (%) | 1 | 0 |
| CCS | Asymptomatic - n (%) | 0 (0%) | 2 (%) | | |
| | Class I - n (%) | 3 (33.33%) | 2 (%) | | |
| | Class II - n (%) | 1 (11.11%) | 1 (%) | 0.68 | 0 |
| Left Ventricular Ejection Fraction | Good (>49%) - n (%) | 5 (50%) | 4 (%) | | |
| | Fair (30-49%) - n (%) | 0 (0%) | 1 (%) | 1 | 0 |
| Extent of coronary disease | Normal/ 1VD - n (%) | 2 (20%) | 3 (%) | | |
| | 2VD - n (%) | 1 (10%) | 0 (%) | | |
| | 3VD - n (%) | 2 (20%) | 2 (%) | 1 | 0 |
| Pre-operative PaO2/FiO2 ratio - Median (IQR) | | 371.05 (188.78) | 475.24 (150.22) | 0.36 | 0 |
| Pre-operative Serum Creatinine (umol/L) - Median (IQR) | | 79.6 (10.62) | 83.8 (22.35) | 0.72 | 0 |
| Pre-operative Serum Troponin (ng/mL) - Median (IQR) | | 79.6 (10.62) | 83.8 (22.35) | 0.72 | 0 |
| Pre-operative Serum NT-proBNP (pg/mL) - Median (IQR) | | 14 (16.2) | 12 (13.6 | 0.79 | 0 |
| Pre-operative MODS - Median (IQR) | | 0.8 (1.79) | 0.8 (0.45) | 1 | 0 |
| Pre-operative Lactate - Median (IQR) | | 1.12 (0.62) | 0.98 (0.32) | 0.69 | 1 |
| Post-operative Lactate (at return to ICU) - Median (IQR) | | 2 (1.6 - 2.2) | 1.9 (1.7 - 2.1) | 0.75 | 0 |
| CBP time | | 131.6 (66.56) | 119 (18.8) | 0.7 | 0 |
| Cross-clamp time | | 70 (39.93) | 75.2 (16.77) | 0.8 | 0 |

**Table S2 – miR selection**

for verification with qRT-PCR.

| miR | Source | Detected in urine |
|---|---|---|
| miR-10a-5p | This study | Yes |
| miR-26a-2-3p | This study | Not detected |
| miR-26a-2-5p | This study | Not detected |
| miR-196a-3p | This study | Not detected |
| miR-196a-5p | This study | Yes |
| miR-93-3p | This study | Not detected |
| miR-93-5p | This study | Yes |
| miR-99a-3p | This study | Yes |
| miR-99a-5p | This study | Yes |
| miR-103b | This study | Not detected |
| miR-21-3p | [6-8] | Not detected |
| miR-21-5p | [6-8] | Yes |
| miR-125a-5p | Sullo et al., 2018 | Yes |

**References**


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