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

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Research Article

Keywords: Surgical Procedure, Cardiovascular, Cell-Derived Microparticles, MicroRNAs, Acute Kidney Injury, Biomarkers

Posted Date: February 4th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1273325/v1>

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Abstract

Background

Secretion of extracellular vesicles (EV) and associated micro-RNAs (miR) is altered during cellular stress and may serve as biomarkers of organ injury. We hypothesized that measuring changes in urinary levels of EV and miR will predict the onset of acute kidney injury in cardiac surgery patients.

Methods

In this predictive accuracy biomarker study performed in the cohort of the REVAKI-2 trial, urine samples were collected before and 24 hours after the procedure from 93 cardiac surgery patients. Urine EV concentrations and size distribution were assessed using NanoSight. EV derivation and levels were measured using flow cytometry. Samples from 10 selected patients were sequenced to detect differentially expressed miR. Verification was performed with advanced TaqMan assays in samples from all patients.

Results

Urine EV concentrations significantly increased in patients with AKI after surgery, with the percentage of EV positive for aquaporin-2 and β 1-integrin also increasing. Pre-surgery podocalyxin-positive EV were significantly lower, and β 1-integrin EV were higher in patients with AKI. The levels of the former correlated with the severity of the injury. miR-125a-5p was expressed at higher levels in urine from patients with AKI stage 2/3. Levels of miR-10a-5p decreased after surgery in AKI patients; its levels correlated with the severity of the injury. Preoperative levels of podocalyxin EVs and miR-125a-5p had moderate AKI predictive value and, in a logistic model together with ICU lactate levels, offered good (AUC = 80.9%) AKI prediction.

Discussion

Urine EV concentrations and miR expression offer excellent predictive accuracy when combined with commonly measured biomarkers.

Introduction

Acute kidney injury (AKI) is a common complication of cardiac surgery affecting up to 30% of patients. Patients who develop AKI suffer from increased morbidity and are four times more likely to die in hospital. Patients with AKI also need longer hospitalization, which increases healthcare costs. [1–3] AKI is defined by an acute drop in glomerular filtration rate as a result of injury to epithelial cells in renal tubules. Routine monitoring of the injury includes measuring urine output and the accumulation of

circulating creatinine, a by-product of muscle metabolism. However, serum creatinine lacks diagnostic and prognostic accuracy, and the development of new and better biomarkers (e.g. Dickkopf [4] and TIMP2/IGFBP7 [5]) has been the subject of intensive research in recent years. However, the pathogenesis of AKI is still poorly understood, many novel and established biomarkers are affected by underlying conditions, and no biomarker has emerged as the AKI gold standard for diagnosis or risk stratification. [6]

Extracellular vesicles (EV) are lipid membrane-bound structures less than 1 µm in size. The EV are classified as microvesicles that bud off the plasma membrane or exosomes that are secreted through the exocytic pathway. EV contain proteins specific for their origin; therefore, changes in specific EV's concentration during pathological processes can point towards affected cells or tissues (reviewed in [7]). In addition, EVs contain different types of RNA molecules that can influence gene expression in the target cells, and as such, are means of intracellular communication. These include messenger RNA, microRNA (miR) and other non-coding RNA. miRs are short (19 – 23) oligonucleotides that regulate post-transcriptional gene expression. Several of them contribute to AKI (reviewed in [8]), and some plasma derived miR have been shown to be AKI biomarkers in children [9] and adults [10].

We hypothesized that urinary EV and miR levels differ at baseline or post-cardiac surgery in people who progress to AKI. The aim of this study was to establish whether these markers may serve as novel predictive or diagnostic biomarkers of AKI.

Materials And Methods

Study design Predictive accuracy biomarker study performed in the cohort of the REVAKI-2 trial (registration ISRCTN18386427) that tested the effect of sildenafil (REVATIO®) on post-cardiac surgery acute kidney injury. The trial was a single center parallel-group randomized clinical trial that has been published previously. [11] All participants in REVAKI-2 provided written informed consent to participate in the trial before their operation. The trial complied with the Declaration of Helsinki. The Yorkshire and The Humber Leeds East Research Ethics Committee approved the study (reference 15/YH/0489) on December 7, 2015. The University of Leicester was the trial sponsor.

Study cohort included adult patients undergoing coronary artery bypass graft (CABG), open valve, or combined CABG and open-valve surgery who were at increased risk of developing AKI, as determined by a modified AKI risk score [1] (a predicted risk score of 20% equates to a positive predicted value for developing AKI of >55%) were eligible. Patients with pre-existing AKI, sepsis, Stage 5 chronic kidney disease (CKD), severe hepatic impairment, allergy to PDE Type 5 inhibitors, recent treatment with cytochrome P450 3A4 inhibitors or guanylate cyclase stimulators were excluded.

Blood and urine sampling Blood was collected before surgery and 6-12, 24, 48, 72 and 96hrs after surgery (citrate and serum). Urine was collected before surgery and 24hrs after.

Outcomes AKI was determined according to the Kidney Diseases Improving Global Outcomes (KDIGO) definition. [12] Other outcomes included plasma levels of troponin and NT-proBNP, urinary levels of

TIMP2/IGFBP, NGAL, creatinine, total urinary EV concentrations, fractions of EV positive for podocalyxin, aquaporin 2, NHE3, CD143, CD144, activated β 1-integrin (HUTS21) and CD29; levels of miR as defined by the sequencing analysis, and levels of selected miRs (**Table S1**) measured using real-time PCR technology. Details of biomarker measurements are in the Supplementary Material.

Statistical analysis

All data were analyzed with R software for statistical computing and graphics [13]. Normally distributed continuous variables are reported as mean (standard deviation, SD). Variables not normally distributed are reported as median (interquartile range, IQR). Parametric comparisons between groups were performed using Welch's t-test or ANOVA. Non-parametric comparisons were performed with the Kruskal-Wallis test or test of equality between regression curves (*sm.ancova*, R). All EV and miR data were analyzed unadjusted and adjusted for either urinary creatinine or total EV concentrations. Models for multiple variables were built by binomial logistic regression and goodness of fit was performed using the Hosmer and Lemeshow test. The area under the curve was calculated with *pROC* R-package.

Results

Between September 2015 and September 2018, 1062 patients undergoing cardiovascular surgery were screened for participation in the REVAKI-2 trial [11]. Three hundred nine were eligible, out of which 180 declined consent, resulting in 125 included patients. Sixty-nine patients developed post-surgery acute kidney injury, and 56 did not. The analysis population included 45 patients with AKI and 49 without (Figure 1A). The average age was 74 (AKI) and 72 (non-AKI), of which 11 and 6% were females. The AKI groups were well matched apart from preoperative serum troponin I and lactate levels measured at the return from the theatre, both higher in the AKI group. Patients with AKI also had significantly higher levels of postoperative acute lung injury and multiple organ injury score. Levels of missing data were low, and less than 10% for all values but pre-operative troponin (Table 1). The most common was AKI stage 1 (n = 36), followed by stage 2 (n = 8) and stage 3 (n = 1).

Table 1

– **Pre- and Postoperative characteristics in the analyzed 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 = 94		No AKI	AKI	P-value*	Missing data (n)
Age (years) - Median (IQR)		74 (70 - 78)	71 (66 - 75)	0.12	0
Sex (female) - n (%)		11 (11.7%)	6 (6.38%)	0.29	0
Ethnic (Caucasian) - n (%)		49 (52.13%)	43 (45.74%)	0.23	0
Sildenafil intervention		25 (26.6%)	20 (21.28%)	0.54	0
Diabetes - n (%)		14 (14.89%)	22 (23.4%)	0.06	0
Stroke/Transient Ischaemic Attack - n (%)		0 (0%)	1 (1.08%)	0.48	1
Chronic obstructive pulmonary disease - n (%)		4 (4.26%)	3 (3.19%)	1	0
Renal disease - n (%)		7 (7.29%)	0 (0%)	1	0
Myocardial infarction - n (%)		19 (19.79%)	0 (0%)	0.35	0
Pulmonary hypertension - n (%)		12 (12.5%)	0 (0%)	0.59	0
Anemia n (%)		21 (22.83%)	24 (26.09%)	0.4	2
Surgery type	CABG - n (%)	18 (19.15%)	17 (18.09%)	0.87	0
	Valve - n (%)	12 (12.77%)	14 (14.89%)		
	CABG & Valve - n (%)	15 (15.96%)	11 (11.7%)		
	other - n (%)	4 (4.26%)	3 (3.19%)		
NYHA	Class I - n (%)	6 (6.67%)	5 (5.56%)	0.95	4
	Class II - n (%)	33 (36.67%)	29 (32.22%)		
	Class III, IV - n (%)	8 (8.89%)	9 (10%)		
CCS	Asymptomatic - n (%)	17 (18.89%)	11 (12.22%)	0.7	4
	Class I - n (%)	13 (14.44%)	14 (15.56%)		

n = 94		No AKI	AKI	P-value*	Missing data (n)
	Class II - n (%)	14 (15.56%)	16 (17.78%)	0.13	1
	Class III, IV - n (%)	3 (3.33%)	2 (2.22%)		
Left Ventricular Ejection Fraction	Good(>49%) - n (%)	38 (40.86%)	28 (30.11%)	0.13	1
	Fair(30-49%) - n (%)	9 (9.68%)	13 (13.98%)		
	Poor(<30%) - n (%)	1 (1.08%)	4 (4.3%)		
Extent of coronary disease	Normal/ 1VD - n (%)	21 (22.58%)	23 (24.73%)	0.46	1
	2VD - n (%)	5 (5.38%)	7 (7.53%)		
	3VD - n (%)	22 (23.66%)	15 (16.13%)		
Pre-operative PaO2/FiO2 ratio - Median (IQR)		457.14 (409.52 - 533.33)	457.14 (409.52 - 533.33)	0.7	2
Pre-operative Serum Creatinine (umol/L) - Median (IQR)		83 (72 - 97)	92 (79 - 111)	0.07	0
Pre-operative Serum Troponin (ng/mL) - Median (IQR)		10 (7.8 - 20.4)	20 (10 - 36)	0.01	14
Pre-operative Serum NT-proBNP (pg/mL) - Median (IQR)		6 (5.9 - 12.2)	10 (10.1 - 25.9)	0.31	4
Pre-operative MODS - Median (IQR)		0 (0 - 1)	1 (0 - 1)	0.05	5
Pre-operative Lactate - Median (IQR)		1 (0.8 - 1.5)	1.35 (1 - 1.63)	0.09	4
Post-operative Lactate (at return to ICU) - Median (IQR)		1.9 (1.58 - 2.6)	2.6 (2.02 - 3.58)	<0.01	0
CBP time		102 (72 - 122)	102 (86 - 134)	0.22	0
Cross-clamp time		65 (46 - 80)	63 (52 - 85)	0.67	0

Serum creatinine levels peaked 48 hours after surgery and were highest in patients with AKI stages 2 and 3. Urinary creatinine displayed a reverse relationship 24 hours after the surgery. There were no differences between AKI/ no AKI with respect to urinary levels of NGAL and TIMP2/IGFBP7, although concentrations of both markers increased 24 hours after surgery (Figure 1B).

There was no difference in total EV concentration between AKI groups, although post-surgery concentrations increased with AKI severity reaching borderline significance of 0.05 (Figure 2A, **left panel**). Very small particles (<100 nm) were significantly more populous pre-surgery in patients without AKI, while particles greater than 300nm in size were significantly more numerous in patients with AKI stages 2 and 3. Particles 50 – 120nm and 50-200 nm were concentrated significantly higher after surgery in patients with AKI stage 2/3 and stage 1, respectively. In addition, numbers of particles greater than 200nm increased in samples from patients with stage 2/3 AKI (Figure 2A, **right panel**).

Flow analysis included exosomes positive for AQP2 (derived from collecting ducts [14]), NHE3 (expressed in renal proximal tubular cells [15]) and PODXL (specific for podocytes in the glomerulus [16]). Microvesicles were tested with antibodies against β 1-integrin (total – CD29, and activated - HUTS-21), epithelial markers (CD105, CD143 and CD144) and toll-like receptor 4 (CD284). A summary of the unadjusted and adjusted EV analyses is shown in Figure 2B. PODXL-positive exosomes significantly decreased in patients with AKI and were lowest in patients with stage 2/3. The differences were apparent in unadjusted and EV or urinary creatinine-adjusted data ((Figure 2C and **D**)). Patients with AKI had a significantly higher percentage of microvesicles positive for activated β 1-integrin (adjusted for MV concentrations, Figure 2E) before and after surgery. The percentage of AQP2-positive microvesicles, adjusted for EV concentrations, was significantly higher post-surgery in patients with AKI (Figure 2F). Fractions of CD144-positive microvesicles significantly increased in patients with stage 2/3 before surgery in unadjusted and EV concentrations-adjusted data (Figure 2G). Finally, post-operative percentages of CD284 microvesicles adjusted for urinary creatinine were lower in patients with stage 2/3 AKI (Figure 2H).

To identify AKI-specific miR, we sequenced RNA isolated from urinary extracellular vesicles. The analysis included five patients with AKI and five without. To avoid heterogeneity in such a small cohort, we only selected patients without diabetes or CKD. Other characteristics were also well matched (**Table S1**). The sequencing data analysis identified six miR: miR-99a and 93 were lower in AKI patients before surgery and across all time points. miR-103b-2 was specifically lower in patients with AKI before surgery, and miR-26a-2, 196a-2 and 10a-5p were significantly lower in patients with AKI after surgery (Figure 3A). Apart from miR-10a-5p, the sequencing analysis identified stem loops of miRs rather than mature miR. Therefore, in the following qRT-PCR verification, we included both mature strands for each miR stem loop. We also tested the expression of miRs previously identified in children [9] and miR-21, which is the most often reported urinary miR (**Table S2**). [17–19] From this list, eight miRs were detectable in the majority of the samples. Two miRs showed differential expression levels when comparing AKI groups (Figure 3B). AKI patients had significantly lower levels of miR-10a-5p after surgery when unadjusted and EV concentrations-adjusted (Figure 3C) data was analyzed. Post-surgery levels of miR-10a-5p decreased with AKI severity and were lowest in patients with stage 2/3, particularly after adjusting for EV concentrations (Figure 3D). miR-125a-5p expression before surgery decreased significantly in patients with AKI when analyzing EV concentrations adjusted data (Figure 3E). However, it increased in patients with AKI stage 2/3 when EV concentration adjusted, and unadjusted data was analyzed (Figure 3F).

None of the five significantly different variables pre-operatively and ICU lactate correlated with each other (Figure 4A). Post-operatively, levels of EV positive for AQP2 significantly correlated with EV concentration, activated β 1-integrin-positive EV and urine creatinine. In addition, EV positive for CD284 and activated β 1-integrin correlated with each other and with urinary creatinine (Figure 4B).

To assess the prognostic value of the preoperative variables, we calculated AUC for each of them. Troponin I, lactate and PODXL had AUC 0.63 – 0.70 while EV positive for CD144 and miR-125a-5p had AUC 0.57 and 0.59, respectively (Figure 4C). We fitted a logistic regression model to analyze the relationship between levels of preoperative/ICU variables and the probability of developing AKI. Levels of PODXL, miR-125a-5p and lactate were significant predictors of AKI. Levels of PODX and miR-125a-5p negatively correlated with the probability of AKI (odds ratios 0.08 and 0.62), while the lactate levels measured at the return to ICU correlated positively (odds ratio 1.95; Figure 4D). The logistic model that included the significant variables had a very good predictive value for AKI (AUC = 80.9%; Figure 4E).

Discussion

Levels of miR-125a-5p and EV derived from podosomes, epithelial cells, and expressing activated β 1-integrin differed before surgery in patients with AKI. Out of these, only EV positive for PODXL and miR-125a-5p were predictive for AKI. Together with post-theatre lactate levels, their predictive value was excellent. After surgery, we observed increased overall EV concentrations and EV positive for AQP2 and activated β 1-integrin. In addition, levels of miR-10a-5p were lower in patients with AKI, and the decrease was proportional to the severity of AKI.

Clinical importance

Kidney PODXL express specifically in podosomes, where it builds the glycocalyx. It is essential for podosome differentiation and urine filtration [20]. We observed that levels of PODXL -positive exosomes were lower before surgery in patients who later developed AKI. The decreases were proportionate to the severity of AKI injury. Although the differences were small, they were apparent after adjusting for EV and urinary creatinine concentrations. That reassures us that the observed effect is of biological significance and can indicate a lower capacity of these patients' kidneys to filter out waste products (e.g. creatinine), which lead to kidney injury after a surgical insult. PODXL is shed of podosomes together with apical membrane fragments in patients with nephritis [21] and its increasing concentrations associate with glomerular damage [22]. These reports do not contradict our findings since exosomes are secreted from the exocytic compartment rather than bud-off from the plasma membrane. Instead, lower exosomal PODXL can indicate inefficient protein delivery to the plasma membrane or its lower expression levels in podosomes. Further experiments are necessary to verify this hypothesis.

We also observed increased levels of EV positive for activated β 1-integrin but only when the data was adjusted for EV concentrations. The difference further increased after surgery. β 1-integrin is expressed on most cells in its folded conformation. Its activation facilitates the adhesion of immune cells. Whether the observed rise in EV positive for β 1-integrin correlates with inflammation levels needs further research.

AQP2 positive exosomes also increased after surgery. It is in contrast to some published data where exosomal levels of aquaporins decrease during AKI. The decrease was associated with lower renal expression levels of aquaporins, leading to defects in water handling (reviewed in [23]). However, the reports come from animal models of AKI and may not reflect cardiac surgery-induced AKI. On the other hand, levels of AQP2-positive urinary exosomes increase in patients with diabetic nephropathy. [24] The AQP2 results need to be taken with caution since the difference was only apparent after adjusting for EV concentrations.

Out of all tested miR, only two were statistically different in AKI patients. We previously reported miR-125a-5p to increase 6-12 hours after surgery in plasma of children with AKI. [9] In this study, miR-125a-5p was expressed at higher levels pre-surgery in the urine patients who later developed AKI stage 2/3. It only has a predictive value for AKI when combined with podocalyxin and lactate, as its AUC was less than 60%. However, it may be useful for more severe AKI, although we could not test it in our cohort since only nine patients developed stage 2 or 3. Levels of miR-10a-5p decreased after surgery, and the difference was evident in unadjusted and MV-adjusted data. There is not much evidence for the two identified miR as urinary markers of AKI. miR-10a-5p was previously reported as a circulatory marker of AKI, reaching exceptional predictive scores in a model with nine other miRs. [25] miR-21 previously reported in multiple papers as an AKI biomarker did not differ in our cohort.

We did not observe any difference in TIMP2/IGFBP7 and NGAL in patients with AKI. However, levels of TIMP2/IGFBP7 increase most in patients with AKI stage 2 or 3 [5] and there were only nine such patients in our cohort. Another explanation could be that AKI in the diagnosed patients was mainly due to glomerular damage rather than tubular damage. Low levels of preoperative PODXL -positive exosomes, absence of any difference in NHE3-positive exosomes, and a significant increase in creatinine levels immediately after surgery in patients with stage 1 AKI support that hypothesis. Tubular damage likely happened in patients with AKI stages 2 and 3, forming 20% of AKI patients and not leading to noticeable increases in NGAL or TIMP2/IGFBP7.

Limitations The major limitation of studies into AKI biomarkers is using a suboptimal biomarker to define the injury. Serum creatinine can rise without damage to the kidney, and it is not sensitive enough to detect acute tubular injury. [26] Attempts have been made to redefine AKI using several recently discovered biomarkers like Dickkopf-3 or TIMP/IGFBP7 that also take into consideration serum creatinine and urinary output. [27] That would overcome detection difficulties of what appears like a heterogeneous injury that can be diagnosed only with a panel of biomarkers.

Another limitation is the small cohort of patients selected for sequencing analysis. The consequence of it was a small number of differentially expressed miRs, none of which passed false discovery rate adjustment. However, even despite that, miR-10a-5p appeared significantly different when measured in all patients using qRT-PCR. Finally, the study suffers from flow cytometry limitations to detect EV subtypes [28] even despite being the primary tool for the measurements. That limitation is true for microvesicle (CD144, CD284, HUTS-21 and CD29) analysis. Exosomes (PODXL, AQP2 and NHE3) on the other hand,

were first bound to beads coupled to antibodies against CD81, which can be reliably recognized by flow cytometry.

Conclusions

Our results suggest that patients with existing damage to glomeruli are more likely to develop AKI after surgery. In the clinical settings of this study, the injury can be predicted using a model that includes urinary EV positive for PODXL and miR-125a-5p, combined with lactate levels.

Declarations

Statements and Declarations: Mrs. Kumar, Prof. Murphy and Dr Woźniak received a grant from Zimmer Biomet. Dr Murphy also received grants from Terumo and Baxter. The remaining authors have disclosed that they do not have any potential conflicts of interest. British Heart Foundation (RG/13/6/29947), (CH/12/1/29419) to GJM, MW, TK, and HA; Leicester and Bristol National Institute for Health Research Cardiovascular Biomedical Research Units.

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Figures

Figure 1

(A) CONSORT diagram. (B) Levels of serum creatinine and urinary creatinine, NGAL and TIMP2/IGFBP7. Green asterisks indicate a significant difference between no-AKI and AKI stage 1; blue asterisks indicate a significant difference between no-AKI and AKI stage 2/3.

Figure 2

(A) Total EV concentrations in urine (left panel) and their size distribution (right panel). Green and blue bars indicate a significant difference between no-AKI and AKI stage 1 or stage 2/3, respectively. (B) Summary of statistical analysis of EV using unadjusted, EV or urinary creatinine-adjusted data. Green fields indicate a significant difference in all three datasets, yellow in two and pink in 1 dataset. U – unadjusted data, E – EV concentrations-adjusted data, C – urinary creatinine-adjusted data, N – no

difference. Plots of EV positive for PODXL (**C** and **D**), activated β 1-integrin (HUTS21, **E**), AQP2 (**F**), VE-cadherin (CD144, **G**) and Toll-like receptor 4 (CD284, **H**). EV conc and UCr indicate that the data were adjusted for EV concentrations or urinary creatinine, respectively.

Figure 3

(A) – summary of the sequencing analysis: Venn diagram of miRs significantly different in three comparisons. Graphical representation of log2 fold changes of significantly different miRs. (B) Summary of statistical analysis of qRT-PCR analysis of miR using unadjusted, EV or urinary creatinine-adjusted data. Yellow fields indicate a significant difference in two and pink in 1 dataset. U – unadjusted data, E – EV concentrations-adjusted data, C – urinary creatinine-adjusted data, N – no difference. (C – F) Plots of miR-10a-5p and miR-125a-5p whenever a significant difference was detected between AKI groups and AKI stages. Asterisks indicate a significant difference between AKI groups.

Figure 4

Correlations between biomarkers significantly different between AKI groups before or at the return from the theatre (**A**) and 24 hours after surgery (**B**). (**C**) Predictive value of biomarkers that were significantly different before surgery. (**D**) Details of the logistic regression analysis. OD – odds ratio. (**E**) ROC curve for a logistic model from **D**.

Supplementary Files

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- [SupplementaryMaterial.pdf](#)