An observational cohort feasibility study to identify microvesicle and miRNA biomarkers of acute kidney injury following paediatric cardiac surgery

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Abstract

Objective Micro (mi)RNA, small non-coding RNA fragments involved in gene regulation, and microvesicles (MV), membrane-bound particles <1μm known to regulate cellular processes including responses to injury, may serve as disease specific biomarkers of acute kidney injury (AKI). We evaluated the feasibility of measuring these signals as well as other known AKI biomarkers in a mixed paediatric cardiac surgery population.

Design: Single centre prospective cohort feasibility study.

Setting: Paediatric Intensive Care Unit

Patients Twenty four children (≤17yrs), undergoing cardiac surgery with cardiopulmonary bypass (CPB) without pre-existing inflammatory state, AKI, or extracorporeal life support.

Interventions: None

Measurements and Main Results: AKI was defined according to modified KDIGO criteria. Blood and urine samples were collected pre-operatively and at 6-12 and 24 hours. MV derivation was assessed using flow cytometry and NanoSight analysis. miRNAs were isolated from plasma and analysed by microarray and quantitative real time polymerase chain reaction. Data completeness for the primary outcomes was 100%. Patients with AKI (n=14/24) were younger, underwent longer CPB, and required greater inotrope support. AKI subjects had different fractional content of platelets and endothelial-derived MV before surgery. Platelets and endothelial MV levels were higher in AKI patients. A number of miRNA species were differentially expressed in AKI patients. Pathway analysis of candidate target genes in the kidney suggested that the most often affected pathways were phosphatase and tensin homolog (PTEN) and signal transducer and activator of transcription 3 (STAT3) signalling.

Conclusions Microvesicles and miRNAs expression patterns in paediatric cardiac surgery patients can be measured in children and potentially serve as tools for stratification of patients at risk of AKI.

Introduction

Acute kidney injury (AKI) occurs in 30% to 50% of paediatric cardiac surgery patients where it is associated with increased mortality, morbidity and the use of healthcare resources[1-3] Improving outcomes in patients with AKI is a clinical research priority.[4] Currently, our understanding of the underlying pathogenesis is poor and there are no effective prevention strategies or treatments despite decades of research.[5,6] This is particularly evident in the paediatric population where age-related changes in levels of serum creatinine [7,8] as well as other factors including volume depletion, hypotension, and sepsis have significant impact on the diagnosis and contribute to the apparent lack of consensus on the definition of AKI in children [9]. As such, serum creatinine does not provide sufficient information about the affected tissue, making targeted therapy impossible. Therefore, there is a need for new biomarkers that allow earlier and more accurate diagnosis or risk stratification in children.[10-12]

Plasma derived microvesicles (MV) and micro-RNAs (miRNA) are involved in the regulation of cellular processes in health and disease. MV contain cell membrane derived marker proteins from the cells they are released from and may serve as cell specific biomarkers of tissue inflammation or injury. Levels of circulating miRNA also correlate well with disease-specific changes in affected tissues and their signalling events, which combined with their stability in body fluids, resistance to RNases, high pH, temperatures and extended storage, makes them perfect biomarkers.[13] These characteristics have led to the evaluation of miRNAs' and MV as biomarkers in a range of diseases. For example in cancer patients specific miRNAs have been shown to indicate early recurrence after chemotherapy[14,15].

The utility of MV and miRNA as disease specific biomarkers in paediatric cardiac surgery has not been well explored and only three miRNAs were selectively tested as AKI biomarkers in a previous study [16]. The objectives of the current study were: (a) to evaluate the feasibility of measuring novel biomarkers (plasma miRNA and MV) of AKI before and after cardiac surgery in a

mixed paediatric population.; (b) identify potential AKI pathogenic pathways; (c) evaluate novel methods for AKI risk stratification before surgery; and (d) compare the novel biomarkers with conventional biomarkers as well as measures of processes thought to be involved in AKI (the systemic inflammatory response, and platelet, leucocyte and endothelial activation [17]). We hypothesized that levels of MV and miRNA will differ in children with and without AKI, reflecting key disease processes thought to contribute to post cardiac surgery AKI, and allow stratification of AKI in different patient populations.

Materials and Methods

Detailed methods are described in the online only digital Supplement.

Study design The Microvesicles and miRNA as markers of Acute Kidney Injury following paediatric cardiac surgery (pMiVAKI) was a prospective observational cohort feasibility study. The study protocol was registered at https://clinicaltrials.gov/ct2/show/NCT02289040, and is included as an online appendix. The study was approved by The East Midlands – Nottingham 1 Research Ethics Committee. The study is reported as per the STrengthening the Reporting of OBservational studies in Epidemiology (STROBE) statement[18].

Study cohort included consecutive patients (defined as newborns if ≤30 days old, infants if >30 days and ≤12 months and children if >12 months) who underwent surgery for congenital heart diseases at the East Midlands Congenital Heart Centre, Leicester, UK, between October 2014 and February 2015. Participants were prospectively recruited if they were younger than 18 years old, their body weight was greater than 2 kg and their operation required cardiopulmonary bypass (CPB). Patients with pre-existing inflammatory state, sepsis undergoing a treatment or with a chronic inflammatory disease or acute kidney injury within 5 days before surgery, emergency or salvage procedures and Extracorporeal Membrane Oxygenation (ECMO) were excluded. Written informed consent/assent from parents or legal guardians or patients was obtained before enrolment as specified in the study protocol.

Blood sampling Three sets of blood samples (clotting, EDTA, citrate and hirudin) were collected from each patient pre-operatively, and 6 – 12 and 24 hours after the end of surgical procedure from indwelling arterial lines. Total sample volumes over 24 hr were depended on patient's weight and determined as follows: 2 – 5 kg, 10.8 ml; 5 – 20 kg, 21.6 ml; >20 kg, 28.8 ml.

Outcomes AKI was determined according to the Kidney Diseases Improving Global Outcomes (KDIGO) definition,[11] with a modification of the Stage 3 definition to include a reduction in

estimated GFR using the Schwartz equation as per the pRIFLE criteria, to adjust for the limited ability of small infants to generate high serum creatinine concentrations.[19,20] The time frame for the assessment of AKI was up to 7 days or hospital discharge if earlier. The primary outcome of the study was the feasibility of measuring and comparison between AKI and non AKI patients of the arterial plasma MV and miRNA concentrations at baseline, 6-12 and 24 hours following surgery. Outcome directly derived from miRNA comparisons between AKI and non AKI groups was identification of potential signalling pathways (as listed in Kyoto Encyclopaedia of Genes and Genomes [21]) in kidney before and after surgery that the miRNA may affect. Secondary outcomes included recruitment and dropout rates, protocol adherence defined as the failure to obtain the specified blood volume or urine sample required for analysis at the required time, completeness of data, and completeness of follow up. As a reference for our proposed novel biomarkers we also measured biomarkers of processes considered to have a causal role in post-cardiac surgery AKI including inflammation; urine Neutrophil Gelatinase Associated Lipocalcin (NGAL), serum cytokine (IL-6, IL-8, MCP-1, and TNFα) levels and levels of cell-free haemoglobin, and markers of platelet (P-selectin expression), monocyte (CD64 expression) and endothelial activation (soluble ICAM-1).

Laboratory analysis

MV analysis was performed in citrated plasma samples spun twice at 1500 xg and stored at -80˚C. Concentration and size distribution were estimated using NanoSight NS500, nano-particle tracking device (Malvern Instruments, Malvern, UK). Derivation of MV was determined with fluorescently labelled antibodies against CD-14, CD41, CD144 (all Affymetrix). Samples (20 – 50 µL) were labelled with antibodies at 1:20 dilution in PBS for 25 min at RT in total volume 100 µL. The samples were analysed by flow cytometry (Cyan ADP, Beckman Coulter).

miRNA analysis The miRNA was extracted from serum using the miRNeasy kit (Qiagen miRNeasy Mini Kit). miRNA samples were combined in 7 pools defined by age and AKI (**Supplemental Table 1**). Total RNA (100ng), from each pool, was reverse transcribed using the

TaqMan Multiplex RT set for TaqMan Array Human MicroRNA Panel v2.0 (Applied Biosystems) and Veriti thermal cyclers. Pre-amplified cDNA from each pool was analysed using TaqMan® Array Human MicroRNA Card A v2.0 (Applied Biosystem). Quantitative PCR was carried out on an Applied BioSystems 7900HT thermocycler. The experiments were performed in triplicates.

Any assays where cycling time (Ct) value was lower than 20 were removed ΔCt (difference in cycling threshold) values were calculated (ΔCt = miRNA Ct—Reference miRNA Ct) using as reference control U6 snRNA-001973 present in duplicates on each miRNA array. Undetermined assays were assigned zero expression value. Data were normalized across arrays using quantile normalisation. To associate miRNA, which were significantly different between AKI and non-AKI groups with their target genes, lists of miRNA were subjected to Target Filter (Ingenuity Pathway Analysis software package, Qiagen) with Tarbase 7.0 and TargetScanHuman. The list of the identified target genes was further reduced to those where miRNA regulation was proven experimentally [22] and those where potential regulation was predicted with high confidence [23]. The list of target genes was further filtered for genes encoding proteins in kidney as listed in Human Protein Atlas[24,25]. The filtered lists for before surgery and after surgery in infants and children were subjected to Expression Analysis (Ingenuity Pathway Analysis, Qiagen).

Statistical analysis

The statistical analysis was performed with SPSS 22.0 (SPSS Inc, Chicago, Ill) and R version 3.3.2.[26] Results were considered statistically significant at the 5% level. All continuous variables were tested for normality with the Kolmogorov–Smirnov test and the Shapiro-Wilk test. Non-normally distributed variables were log-transformed. Clinical baseline and perioperative outcomes were analysed with one-way analysis of variance (ANOVA) and chi-square test. Continuous outcomes measured at repeated time points (biomarker concentrations, measures of platelet and leucocyte activation) were analysed using linear regression models and analysis of variance (lm and anova – R, stats-package), and considered time and age as interaction variables with AKI/ no AKI. Statistical

significance of the main and interaction terms was assessed using global F-statistics. In exploratory analyses, where p-value < 0.05 the nature of interactions between AKI status, age and time were inferred by visual inspection of data plots given the small sample sizes in each subgroup. Clustering analysis of miRNA expression profile was performed using hclust program (R, stats package) with WardD2 method and distances were calculated using dist program (R, stats package) using Euclidean method. Heatmaps of miRNA expression profiles were created using pheamap program (R, pheatmap package). In a *post hoc* analysis Pearson correlations and their significance between biomarkers was analysed using cor.test function (R, stats package).

Results

Trial cohort

Between 26th October 2014 and 7th February 2015, 79 patients were screened of which 71 were eligible, 46 were approached out of which 14 patients' surgery was delayed and were not asked for consent. Out of 27 consented patients in 2 cases the surgery was cancelled for clinical reasons and 1 patient withdrew consent. After withdrawals (STROBE diagram in **Supplemental Figure 1A**) the analysis population comprised 5 newborns, 5 infants and 14 children. Average age for newborns was 9 days (range 5.5-13), infants: 242 days (range 147.5-313.5) and for children: 1497 days (range 1046.5-4949). There were 8 cyanotic patients and 44.2% were females. Details of the operations performed are shown in **Supplemental Table 2**. No deaths or need for ECMO support were recorded during the study period. Following cardiac surgery 58.43% of patients developed KDIGO defined AKI; 25% (n=6) were classified as stage 1, 12.5% (n=3) as stage 2 and 20.8% (n=5) as stage 3. AKI was observed in 5/5 newborns, 3/5 infants and 6/14 children. As expected, serum creatinine significantly increased in the AKI group (as indicated by significant AKI, $p=2x10^{-9}$ and AKI*time, p=0.04 terms) and was dependent on patients' age group as indicated by significant interactions with age (p=0.001 **Supplemental Figure 1B and Supplemental Table 4**) with highest levels in the newborns. AKI patients were more likely to be younger, smaller, and to undergo redo procedures or have longer CPB times (**Table 1**). Key perioperative characteristics in AKI and non-AKI patients are reported in **Table 2**.

Protocol compliance Clinical follow up was completed in all patients and assessment of the primary outcome was completed in 94.4% of samples. In some patients it was not possible to collect blood (n = 4 samples: 1 sample before surgery, 1 sample at 6-12 hr and 2 samples at 24 hr after surgery) or urine samples (n = 5 samples: 2 samples before surgery, 1 sample at 6-12 hr and 2 samples at 24 hr after surgery) because of patient's discomfort or early removal of indwelling lines and urine catheter. No major protocol violations or adverse events related to study procedures were

recorded. Sample collection time was adherent to protocol, except for 3 samples. Laboratory and data analysis completeness are indicated in **Supplemental Figure 1A**.

Primary outcomes

Microvesicles: There was no overall difference in MV concentrations between AKI and non-AKI patients (**Figure 1A**). The MV were of uniform ~180 nm size and there were no significant differences in either distribution or concentrations (**Figure 1B**) between patients who developed AKI and those who did not, although a significant interaction was observed between AKI status and patients' age (P value for interaction 0.01). Exploratory analyses further detected significant differences between age groups (p-value = 1.0×10^{-3}) and inspection of data plots suggested that MV concentrations were higher in children and lower in infants with AKI (**Figure 1A, B**). Analysis of MV derivation using flow cytometry (**Figure 1C-E** and **Supplemental Table 3**) identified significantly higher levels of platelet derived and endothelial cells derived MV in AKI patients (p-values = 1.49×10^{-4} and 4.96×10^{-5}, respectively). No interaction with time or age group was present. No difference was observed for monocyte-derived MV in AKI patients.

miRNA analysis: Analysis of expression levels indicated that pre-surgery miRNA levels were shown to be most different between AKI and non AKI in children and in infants (pre; **Figure 2A – B**). An increase in concentrations at 6-12 hours after surgery was also observed for some miRNA clusters (**Figure 2A**). This was most evident for miRNA expression patterns averaged across samples from all patients in samples from both all patients and children only (Figure 2A, first two heatmaps). In infants some miRNA clusters showed an opposite pattern where their expression was lower after surgery, as compared with other time-points. Strong time dependence was confirmed by clustering analysis: Expression patterns at either 6-12 hr or 24 hr time-points clustered together regardless of the presence of AKI indicating that surgery has a stronger effect on miRNA expression than the presence of AKI (**Figure 2B**). Again, this was most evident for children expression patterns and when

all patients' data were considered. The clustering analysis also indicated that miRNA expression patterns for AKI and non AKI groups were most different before surgery (**Figure 2B**).

Differential miRNA expression between AKI and non-AKI groups in samples from patients before and after surgery are reported in **Table 3**. Three miRNAs were found to be significantly different in children and infants with AKI (hsa-miR-7g-5p, hsa-miR-152-3p and hsa-miR-320a). However while hsa-miR-7g-5p and hsa-miR-152-3p were upregulated in infants with AKI, their expression levels were lower in children with AKI as compared with non-AKI patients. For hsa-miR-320a, an opposite pattern was evident. Target genes of differentially expressed miRNAs were identified to explore affected signalling pathways as described in Materials and Methods. The top five most likely affected canonical pathways and genes involved in renal toxicity are reported in **Table 4**. The most often affected pathways were PTEN (phosphatase and tensin homolog) and STAT3 (signal transducer and activator of transcription 3) signalling, particularly after surgery. Most often targeted genes before surgery included: VIM, targeted by 5 miRs, and AGO4, ARFIP1, LIN28A, YPEL2, ZNF226 each targeted by 4 miRs. Most often targeted genes after surgery included: LCOR, targeted by 5 miRs, and AGO4, DDX19B, DICER4, each targeted by 4 miRs.

Secondary Outcomes

Urine NGAL was higher in AKI versus non-AKI patients, and this effect did not interact with age group (P-value for AKI*age gr. interaction > 0.05; **Supplemental Table 4**). There were mixed changes in serum cytokine markers of the systemic inflammatory response: from IL-8, MCP-1, TNFα and IL-6, only the first three were significantly different in patients with and without AKI. For IL-8 a significant interaction AKI*time was observed (p-value = 3.0×10^{-3}) and its levels were highest 6 hr after surgery in AKI patients, overall MCP-1 levels were significantly higher (p-value = 0.04) and TNFα lower (p-value = 1.82×10^{-4}) in AKI patients. TNFα also showed significant AKI*age gr interaction (p-value = 0.01; **Supplemental Table 4**). Platelets and monocyte activation assessed using flow cytometry were significantly higher in AKI patients (p-value = 0.01 for both; **Supplemental Table 4**).

No significant interaction with time was identified for these outcomes, suggesting that these may reflect general differences in AKI susceptibility. Levels of cell free haemoglobin where higher in AKI patients, with significant interaction between AKI status and age (**Supplemental Table 4**). Evaluation of the plots suggested that cell free haemoglobin was not elevated in neonates with AKI. Serum ICAM, a marker of endothelial activation was not different between groups (**Supplemental Table 4)**. The majority of markers measured as secondary outcomes did not show any significant correlation with any microvesicle variables, except leukocyte activation, which correlated significantly with microvesicles derived from platelets ($p = 0.02$, $r = 0.31$) and from endothelial cells (p-value = 7.76×10^{-4}). Platelet and endothelial cells-derived microvesicles also significantly correlated with each other (p-value = 5.90×10^{-5}, $r = 0.47$). Correlation analysis is shown in **Supplemental Table 5**.

Discussion

Microvesicles and miRNAs levels are detectable in paediatric cardiac surgery patients of every age, including neonates. Exploratory analyses suggest that different age groups have specific expression patterns depending on the time after surgery, and AKI status. Analysis of microvesicles derivation indicating platelet and endothelial cell activation demonstrated differences between AKI and non-AKI groups and along with the results of the analysis of miRNA changes point toward the potential utility of these biomarkers for patients' stratification before surgery. In addition our analysis indicated phosphatase and tensin homolog (PTEN) and signal transducer and activator of transcription 3 (STAT3) signalling pathways' potential involvement in AKI pathogenesis in humans.

Clinical importance

This study demonstrated the feasibility of miRNA analysis in small blood volumes in paediatric population. Our results did not refute the hypothesis that miRNA may serve as stratification tools in paediatric patients at risk of developing AKI. We observed overall higher concentrations of miRNA after surgery, and miRNA expression heatmaps (Figure 2A) as well as clustering analyses (Figure 2B) suggested that certain miRNAs are released specifically in response to surgery. Indeed these changes were greater and more consistent than the changes observed in miRNA expression between AKI and non AKI groups. This points to the importance of miRNA signalling as part of the host response to surgery, as has been reported previously. [16] Different expression patterns between children and infants with AKI is also suggestive of age-dependent regulation of miRNA release in response to surgery and AKI. Of the differentially expressed miRNAs identified in this study hsa-miR-27-3p, hsa-miR-10a-5p,hsa-miR-7g (let-7-5p family), and hsa-miR-30b-5p (miR-30-5p family) were also identified in two analyses of miRNA AKI biomarkers in adult cardiac surgery patients.[27,28] In contrast we observed no difference for hsa-miR-210-3p, which was reported as an AKI marker in both adult studies.[27,28] This may also indicate heterogeneous disease processes in adults and children.

AKI status had the strongest association with miRNA expression patterns at baseline as indicated by the clustering analysis where miRNA expression patterns at pre for AKI and non-AKI patients were least similar (different clusters in dendrograms in Figure 2B), suggesting a possible risk stratification role for these biomarkers. However, even at baseline, age-dependent miRNA-expression pattern was evident, particularly for three miRNAs (hsa-let-7g-5p, hsa-miR-152-3p and hsa-miR-320a) whose expression was significantly different between AKI and non-AKI groups but showed opposite expression patterns in children and infants. This points to the potential utility of miRNA analysis to identify different AKI phenotypes, even in different paediatric age groups. To verify the clinical utility of these miRNAs a study in a larger cohort of patients is necessary. Third, our findings provide new insights into AKI pathogenesis. Pathway analysis identified PTEN and STAT3 signalling pathways as affected most often after surgery in AKI patients. Hitherto PTEN has been shown to be a key node regulating apoptosis, proliferation and recovery only in rodents.[29,30] Inhibition of PTEN leads to exacerbated renal damage in animal models of AKI triggered by ischemia/reperfusion [31,32] or cisplatin [33] as a result of increased levels of apoptosis, immune cell infiltration and increased levels of proinflammatory cytokines. Activation STAT3 pathway was also shown to have a protective role in AKI since STAT3 deletion leads to more severe ischemia-triggered AKI in a mouse model [34]. The identified miRNAs targeted PTEN and SMAD3 pathways rather than the two proteins, therefore additional research is necessary to determine the role both pathway play in AKI. Other miRNA-affected genes included Transforming Growth Factor (TGF) β receptors and SMAD (Similar To Mothers Against Decapentaplegic) proteins, which are involved in TGFβ signalling and proliferation. These changes are associated with increased expression of vimentin (targeted by 5 miRNA identified in this study), which leads to fibrosis.[35]

Strengths and limitations

Although single miRNA were previously measured in paediatric cardiac surgery patients [16] this is the first study to our knowledge that measured MV and multiple miRNA biomarkers in

children who develop AKI after cardiac surgery. There was excellent compliance with the study protocol indicating the feasibility of this approach; in total there were 148 laboratory measurements for each patient with an overall level of missing laboratory data of 8.1%. The study used microarrays to measure 378 individual miRNAs in pooled samples, with subsequent bioinformatics analysis. Even in a small cohort this has provided new information on the molecular processes underlying this disease. Platform techniques have seen a rapid reduction in cost over recent years and we suggest that the wider application of 'omics' to perioperative organ injury will yield important new knowledge. Another novel finding was the observation of significant differences between AKI and non AKI patients for MV markers of platelet activation that were also evident with direct measurement of these processes using flow cytometry. MV analysis in stored samples may therefore have the potential to supplant direct measurement of these processes; measures of platelet activation typically must be performed within 15 minutes of sample collection which creates logistical challenges in clinical studies. In contrast, significant differences in endothelial-derived CD144-positive MV did not align with levels of ICAM or CD62E-positive MV. One interpretation is that ICAM or CD62E are not good markers of the endothelial injury that underlies AKI in humans[36], highlighting the value of MV biomarkers in exploring underlying mechanisms.

The main limitations of the study were the small sample size, and the heterogeneity with respect to age, range of heart defects and operations. The study results should therefore be interpreted as hypothesis forming rather than definitive. Our analyses did not assess the source of miRNAs, whether exosomal or protein bound, limiting our ability to interpret the possible mechanisms underlying the observed changes. MV concentrations measured using the Nanosight showed significant variability between patients and this may be considered a further limitation. MV derivation was also measured using flow cytometry which is known to have a number of limitations for particles smaller than 0.5 μm[37] - well above the size of MV observed in the study samples. However, flow cytometry remains the primary tool for measurement of MV derivation in clinical samples,[38,39] we observed very little variability between samples relative to total MV concentrations,

and we demonstrated clear differences between groups for MV derivation, supporting this method. Another limitation is the observational study design; we are therefore unable to claim that the MV or miRNA identified in our analysis have a causal role in AKI pathogenesis, versus parallel or consequential processes. We attempted to mitigate this limitation by restricting our bioinformatics analysis to protein networks in the kidney. In addition, our MV derivation analyses targeted processes that have been previously implicated in trials of off- versus on-pump coronary artery bypass surgery as having a causal role in AKI[40] and may not reflect processes in paediatric AKI. A final limitation is the current consensus definition of paediatric AKI used in the study.[10-12] Urinary catheters were placed in all patients but were removed within 24 hours in 5 patients, which may have introduced bias in AKI detection. However this is unlikely as the removal of urinary catheters typically represents a lack of clinical concern about urine output. It is also likely that serum creatinine rises denote different processes in neonates versus 16 year old children; indeed interaction with age was a common finding for most of the processes studied. In the absence of a consensus definition of AKI in neonates at the time the pMIVAKI study was conducted we incorporated pRIFLE criteria that use the Schwartz equation for neonates [41]. New consensus definitions of AKI in neonates have emerged subsequently, however as these remain primarily creatinine/ urine output based their clinical utility remains unclear [42]. We suggest that the wider use of platform techniques, as performed in this study, to more accurately phenotype AKI will address this limitation going forward.

In summary our results demonstrate the feasibility of measuring multiple MV and miRNA in paediatric cardiac surgery patients. These results also point towards a hitherto unreported heterogeneity of molecular signals within children suffering from AKI. Our findings support a programme of work that will define distinct molecular phenotypes and their underlying processes in AKI, and is designed to overcome many of the limitations identified in this feasibility study. We suggest that this strategy will lead to the development of novel diagnostic tests, more accurate risk stratification, and novel reno-protective treatments targeted to precise AKI phenotypes.

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Figure legends

Figure 1 – Platelets and endothelial cells microvesicles increase in AKI patients A – total MV concentration and **B** – MV size distribution in all patients as estimated with NanoSight device. Children who developed AKI had higher concentrations of MV of ~130nm in size, in plasma at any of the analysed time-points. In infants ~130nm MV increased in concentration after surgery but only in patients who did not develop AKI. MV derivation was determined by flow cytometry with specific antibodies for **C** – monocytes (CD14); **D** – platelets (CD41); **E** – endothelial cells (CD144). Values in line plots represent means of percent of positive particles ±SD for AKI (hatched line) versus no AKI (solid line).

Figure 2 – miRNA in patients with AKI is most different before surgery A – Heatmap of clustered means of normalised (quantile) logged concentrations of all analysed miRNA ($2^{CT(\text{cycle threshold})}$) in all patients, children and infants. Clusters of miRNA increase in expression were most evident 6hr after surgery. **B** – Clustering analysis of miRNA expression patterns between age groups and time-points. Height in Y-axis indicates Euclidean distance at which the clusters were defined. In All age groups the first two clusters were defined at height 37.53, which makes expression miRNA patterns 6 hrs after surgery (that separated further at 22.88) clearly different from other patterns that separated at 25.36. In children two clusters were defined at height 46.45, which further separated at 34.66 and 25.18 defining two distinct groups of miRNA profiles. In infants, miRNA expression profile before surgery (pre) clustered away at height 52.07 making it clearly distinct from other miRNA expression patterns that further separated at height 42.53.

Figure 1

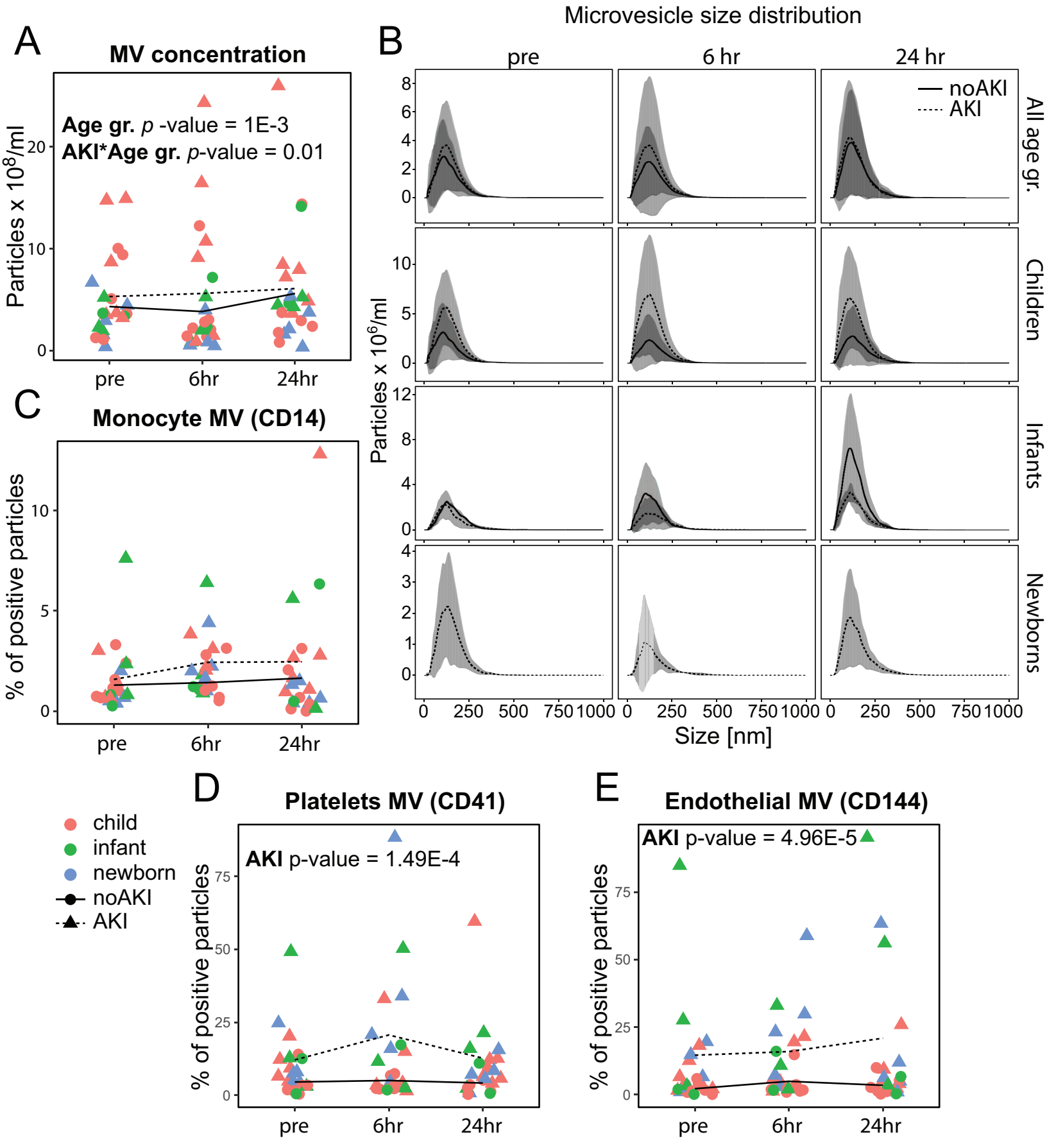
A
MV concentration
Age gr. p -value = 1E-3
AKI*Age gr. p-value = 0.01
Particles x 10^8/ml
pre
6hr
24hr
B
Microvesicle size distribution
pre
6 hr
24 hr
noAKI
AKI
All age gr.
Children
Infants
Newborns
Particles x 10^6/ml
Size [nm]
C
Monocyte MV (CD14)
% of positive particles
D
Platelets MV (CD41)
AKI p-value = 1.49E-4
E
Endothelial MV (CD144)
AKI p-value = 4.96E-5
child
infant
newborn
noAKI
AKI

Figure 2

Figure 2

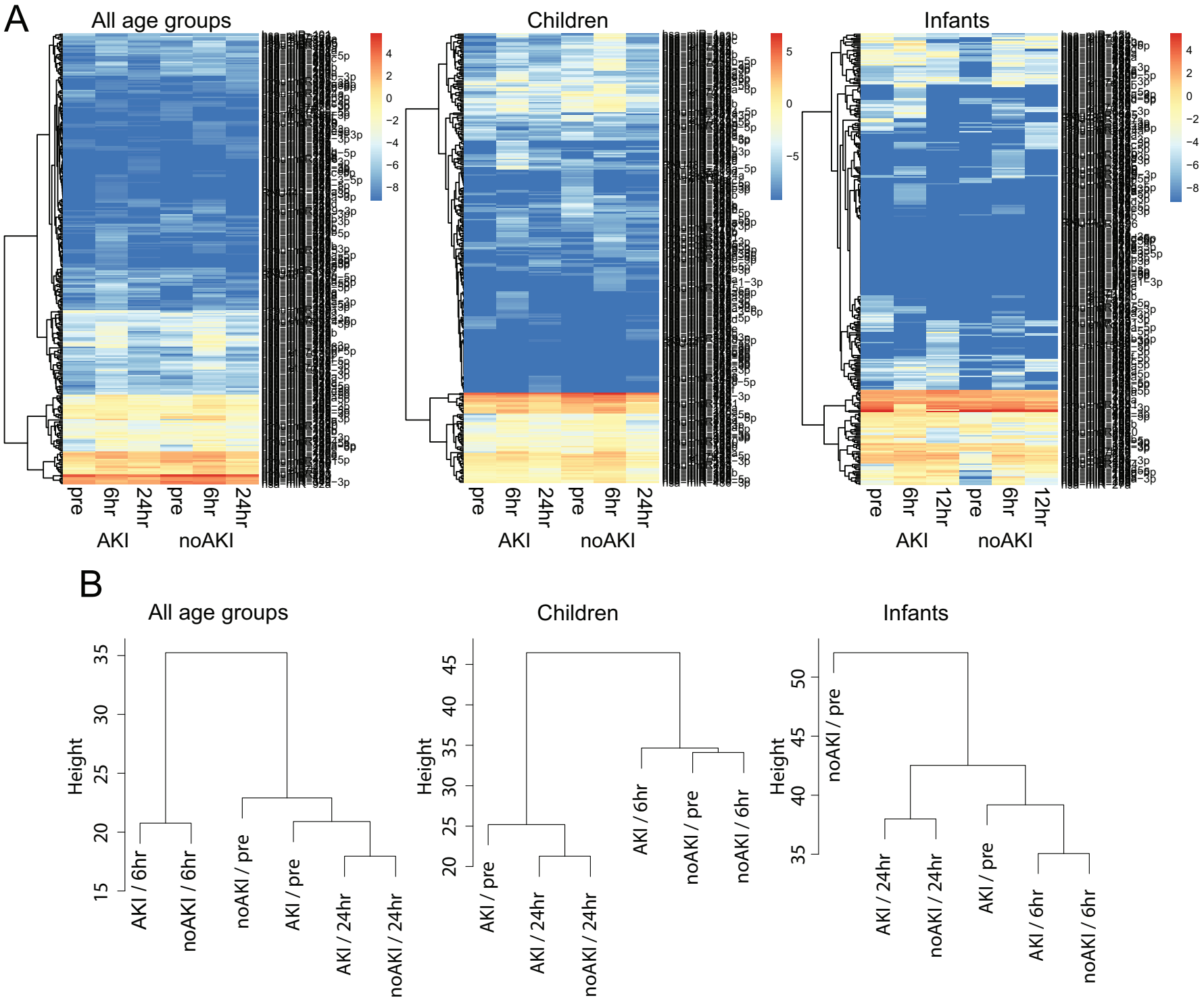

Table 1

Table 1 – Baseline characteristics – Continuous data are presented as mean± standard deviation or median and IQR; categorical variables as number (percent). CPB, cardiopulmonary bypass. RACHS, Risk Adjusted Congenital Heart Surgery Score. *, constant value in the group

Variable	**All age groups (n=24)**			**Children (n=14)**			**Infants (n=5)**			**Newborns (n=5)**		
	AKI (n=14)	**nonAKI (n=10)**	**p-value**	**AKI (n=6)**	**nonAKI (n=8)**	**p-value**	**AKI (n=3)**	**nonAKI (n=2)**	**p-value**	**AKI (n=5)**	**nonAKI (n=0)**	**p-value**
Age (months)	7.85 (0.37-30.43)	46.41 (36.37-90.03)	0.008	38.65 (23.57-169-47)	58.68 (39.0-126.75)	0.529	5.43 (4.92-7.85)	9.35 (8.07-10.63)	0.338	9 (0.18-0.43)	-	-
Height (cm)	61.0 (53.0-85.0)	96.0 (85.0-122.0)	0.089	87.5 (81.0-149.0)	105.0 (90.5-136.5)	0.740	56.0 (56.0-61.0)	63.0*	0.457	52.0 (51.5-53.0)	-	-
Weight (Kg)	7.4 (3.65-11.1)	14.4 (9.2-19.1)	0.036	11.25 (10.1-30.5)	15.45 (13.35-35.05)	0.491	6.8 (5.56-7.41)	6.18 (6.15-6.2)	0.897	3.5 (2.98-3.9)	-	-
Body Surface Area (m2)	0.33 (0.22-0.49)	0.61 (0.46-0.82)	0.022	0.51 (0.48-1.16)	0.67 (0.57-1.15)	0.414	0.3 (0.28-0.33)	0.31*	0.881	0.22 (0.2-0.23)	-	-
Sats (%)	94.0 (91.0-98.0)	96.0 (85.0-99.0)	0.962	95.5 (91.0-99.0)	97.5 (94.0-99.5)	0.862	98.0 (96.5-98.0)	85*	0.007	91.0 (82.5-94.0)	-	-
Cyanotic patients (n,%)	5 (35.7%)	3 (30%)	1.0	1 (16.7%)	1 (12.5%)	1.0	1 (33.3%)	2 (100%)	0.4	3 (60%)	-	-
Males (n,%)	7 (50%)	4 (40%)	0.697	2 (33.3%)	3 (37.5%)	1.0	2 (66.7%)	1 (50%)	1.0	3 (60%)	-	-
Race			0.930			1.0			NA		-	-
Caucasian (n,%)	11 (78.6%)	8 (80%)		5 (83.3%)	6 (75.0%)		3 (100%)	2 (100%)		3 (60%)	-	-
African (n,%)	1 (7.1%)	1 (10%)		0 (0%)	1 (12.5%)		0 (0%)	0 (0%)		1 (20%)	-	-
Asian (n,%)	2 (14.3%)	1 (10%)		1 (16.7%)	1 (12.5%)		0 (0%)	0 (0%)		1 (20%)	-	-

Genetic disease (n,%)	1 (7.1%)	4 (40%)	0.122	1 (16.7%)	4 (50%)	0.301	0 (0%)	0 (0%)	NA	0 (0%)	-	-
Previous cardiac surgery (n,%)	5 (35.7%)	8 (80%)	0.047	4 (66.7%)	7 (87.5%)	0.301	1 (33.3%)	1 (50%)	1.0	0 (0%)	-	-
Preoperative use of diuretics (n, %)	4 (28.6%)	2 (20%)	1.0	1 (16.7%)	1 (12.5%)	1.0	2 (66.7%)	1 (50%)	1.0	1 (20%)	-	-
Preoperative use of prostin (n, %)	3 (21.4%)	0 (0%)	0.239	0 (0%)	0 (0%)	NA	0 (0%)	0 (0%)	NA	3 (60%)	-	-
Total surgery time (min)	292.5 (226.0-390.0)	243.5 (210.0-263.0)	0.045	341.0 (226.0-415.0)	243.5 (190.5-261.0)	0.114	335.0 (284.0-370.5)	236.5 (210.0-263.0)	0.284	270.0 (206.5-330.0)	-	-
Total CPB time (min)	135.5 (114.0-208.0)	105.5 (60.0-127.0)	0.022	184.0 (72.0-275.0)	105.5 (58.5-126.0)	0.113	135.0 (132.5-135.0)	107.5 (65.0-150.0)	0.649	130.0 (110.0-181.0)	-	-
Crossclamp (n, %)	12 (85.7%)	10(100%)	0.493	6 (100%)	8 (100%)	NA	3 (100%)	2 (100%)	NA	3 (60%)	-	-
Crossclamp time (min)	84.5 (36.0-137.0)	53.5 (34.0-89.0)	0.493	88.0 (24.0-198.0)	53.5 (34.5-77.5)	0.852	82.0 (60.0-92.0)	69.5 (34.0-105.0)	0.908	87.0 (73.0-109.5)	-	-
Blood priming (n, %)	14 (100%)	8 (80%)	0.163	6 (100%)	6 (75%)	0.473	3 (100%)	2 (100%)	NA	5 (100%)	-	-
Ultrafiltration (n, %)	12 (85.7%)	8 (80%)	1.0	4 (66.7%)	6 (75%)	1.0	3 (100%)	2 (100%)	NA	5 (100%)	-	-
Circulatory arrest (n, %)	2 (14.3%)	1 (10%)	1.0	2 (33.3%)	1 (12.5%)	0.538	0 (0%)	0 (0%)	NA	0 (0%)	-	-
Return on CPB (n, %)	1 (7.1%)	2 (20%)	0.550	1 (16.7%)	2 (25.0%)	1.0	0 (0%)	0 (0%)	NA	0(0%)	-	-

Lowest temperature (°C)	31.25 (25.4-32.3)	33.4 (31.8-35.4)	0.022	25.35 (25-31.5)	32.0 (31.65-34.9)	0.005	31.0 (29.5-31.35)	36.25 (35.9-36.6)	0.027	33.9 (31.2-35.0)	-	-
Use of cell saver (n, %)	3 (21.4%)	6 (60%)	0.092	2 (33.3%)	4 (50%)	0.627	0 (0%)	2 (100%)	0.1	1 (20%)	-	-
Chest open (n, %)	5 (35.7%)	0 (0%)	0.053	1 (16.7%)	0 (0%)	0.429	0 (0%)	0 (0%)	NA	4 (80%)	-	-
RACHS-1	2	1		2	1		0	0		0	0	
RACHS-2	4	4		0	2		3	2		1	0	
RACHS-3	4	5		2	5		0	0		2	0	
RACHS-4	3	0		2	0		0	0		1	0	
RACHS-6	1	0		0	0		0	0		1	0	

Table 2

Table 2 - Perioperative characteristics – Continuous data are presented as mean± standard deviation or median and IQR; categorical variables as number (percent). PIM2, Paediatric Index of Mortality 2 Score. PICU, Paediatric Intensive Care Unit. CPAP, Continuous positive airway pressure. RBC, red blood cells. *, constant value in the group

Variable	All age groups (n=24)			Children (n=14)			Infants (n=5)			Newborns (n=5)		
	AKI (n=14)	nonAKI (n=10)	p-value	AKI (n=6)	nonAKI (n=8)	p-value	AKI (n=3)	nonAKI (n=2)	p-value	AKI (n=5)	nonAKI (n=0)	p-value
PIM2 score	1.95 (1.7-3.4)	1.55 (1.4-1.8)	0.026	1.85 (1.7-2.0)	1.5 (1.6-3.35)	0.029	2.4 (2.1-2.9)	2.0 (1.6-2.4)	0.485	2.7 (1-3.6)	-	-
PICU admission lactates (mmol/L)	3.6 (2.0-6.4)	2.05 (1.6-2.9)	0.103	4.8 (3.0-9.3)	2.5 (1.6-3.35)	0.181	2.0 (5.0-9.0)	1.25 (1.0-29.0)	0.278	5 (2.9-7.8)	-	-
Intubation time (hours)	38.75 (12.7-96)	4.9 (2.83-17.25)	0.026	10.92 (4.91-29.9)	4.92 (2.33-12.0)	0.282	32.0 (22.38-38.75)	265.42 (2.83-528.0)	0.535	115 (83.8-271.6)	-	-
Reintubation (n, %)	2 (14.3%)	1 (10%)	1.0	1 (16.7%)	1 (12.5%)	1.0	1 (33.3%)	0 (0%)	1.0	0 (0%)	-	-
Reoperation (n, %)	8 (57.1%)	1 (10%)	0.033	3 (50%)	1 (12.5%)	0.245	1 (33.3%)	0 (0%)	1.0	4 (80%)	-	-
12 Hours blood loss (ml/kg/hr)	1.45 ± 0.59	0.97 ± 0,47	0.045	1.24 ± 0.75	0.87 ± 0.47	0.29	1.71 ± 0.41	1.40 ± 0.03	0.334	1.56 ± 0.4	-	-
RBC transfusions (ml/kg)	92.65 ± 47.98	29.66 ± 20.37	0.001	53.74 ± 19.94	23.05 ± 16.84	0.018	82.01 ± 19.25	56.11 ± 0.21	0.169	139.5 ± 44.4	-	-
Vasoactive Inotrope score	14.45 (11.66-26.5)	8.55 (3-11)	0.009	17.13 (10.6-26.5)	8.55 (3.0-10.5)	0.081	13.5 (10.5-21.75)	10.25 (6.0-14.5)	0.518	14.9 (13.3-23.7)	-	-
PICU stay (days)	5.5 (4.0-7.0)	1.0 (1.0-2.0)	0.004	4.5 (2.0-7.0)	1.0 (1.0-2.0)	0.043	5.0 (5.0-9.0)	15.0 (1.0-29.0)	0.443	6 (4.5-14)	-	-
Hospital stay (days)	10.5 (8.0-22.0)	6.0 (6.0-6.0)	0.03	9.0 (6.0-12.0)	6.0 (5.5-9.0)	0.414	8 (8.0-15.0)	6*	0.055	12 (9.5-27)	-	-
Cardiac arrest (n, %)	0 (0%)	1 (10%)	0.417	0 (0%)	0 (0%)	NA	0 (0%)	1 (50%)	0.4	0 (0%)	-	-

Permanent pacemaker (n, %)	1 (7.1%)	1 (10%)	1.0	1 (16.7%)	1 (12.5%)	1.0	0 (0%)	0 (0%)	NA	0 (0%)	-	-
Use of vasodilator (n, %)	5 (35.7%)	1 (10%)	0.341	1 (16.7%)	0 (0%)	0.429	2 (66.7%)	1 (50%)	1.0	2 (40%)	-	-
Mask CPAP (n, %)	3 (21.4%)	0 (0%)	0.239	2 (33.3%)	0 (0%)	0.165	1 (33.3%)	0 (0%)	1.0	0 (0%)	-	-
Pleural drainage (n, %)	3 (21.4%)	2 (20%)	1.0	0 (0%)	1 (12.5%)	1.0	2 (66.7%)	1 (50%)	1.0	1 (20%)	-	-
Neurological event (n, %)	1 (7.1%)	0 (0%)	1.0	1 (16.7%)	0 (0%)	0.429	0 (0%)	0 (0%)	NA	0 (0%)	-	-
Phrenic nerve palsy (n, %)	3 (21.4%)	0 (%)	0.239	1 (16.7%)	0 (0%)	0.429	2 (66.7%)	0 (0%)	0.4	0 (0%)	-	-
Antibiotics used (n, %)	9 (64.3%)	3 (30%)	0.214	3 (50%)	2 (25%)	0.580	3 (100%)	1 (50%)	0.4	3 (60%)	-	-

Table 3

Table 3 – Differentially expressed miRNAs Difference in miRNA expression levels between patients who developed AKI during cardiac surgery and those who did not was analysed using linear regression models with time incorporated as an effect. Whenever the model was statistically significant between AKI and non-AKI patients or when the interaction between AKI and time was significant, the difference between AKI and non-AKI groups was further tested before and after surgery and Log expression ratio and p-value are reported. Positive expression ratio (log Expr ratio) indicates upregulation in AKI and negative expression ratio indicates downregulation.

Age group	Before surgery	miRNA family	Log Expr Ratio	*p*-value	After surgery	miRMA family	Log Expr Ratio	*p*-value
all age groups	hsa-miR-192-5p	miR-192-5p/215-5p	-0.890	0.040	hsa-miR-10a-5p	miR-10-5p	-5.190	0.006
	hsa-miR-487a-3p	miR-154-3p/487-3p	2.450	0.006	hsa-miR-149-5p	miR-149-5p	no expr. In no-AKI	0.006
	hsa-miR-490-3p	miR-490-3p	4.660	0.017	hsa-miR-196b-5p	miR-196-5p	no expr. In no-AKI	0.011
	hsa-miR-501-3p	miR-501-3p/502-3p	0.480	0.040				
children	hsa-let-7g-5p	let-7-5p/98-5p	-3.267	0.041	hsa-let-7g-5p	let-7-5p/98-5p	0.177	0.030
	hsa-miR-152-3p	miR-148-3p/152-3p	-2.887	0.045	hsa-miR-125a-3p	miR-125a-3p	5.924	0.018
	hsa-miR-30b-5p	miR-30-5p	-2.068	0.025	hsa-miR-125a-5p	miR-125-5p	4.886	0.003
	hsa-miR-320a	miR-320	1.319	0.028	hsa-miR-223-3p	miR-223-3p	1.652	0.026
	hsa-miR-484	miR-484/3155	1.053	0.027	hsa-miR-301-3p	miR-130-3p/301-3p/454-3p	5.122	0.042
	hsa-miR-885-5p	miR-885-5p	-1.209	0.046				
infants	hsa-let-7g-5p	let-7-5p/98-5p	18.559	0.007	hsa-let-7g-5p	let-7-5p/98-5p	1.248	0.044
	hsa-miR-125b-5p	miR-125-5p	19.916	0.001	hsa-miR-103-3p	miR-103-3p/107	1.957	0.018
	hsa-miR-133a-3p	miR-133a-3p.2/133b	18.398	0.007	hsa-miR-15a-5p	miR-15-5p/16-5p/195-5p/424-5p/497-5p	1.018	0.038

	hsa-miR-150-5p	miR-150-5p	-1.172	0.013	hsa-miR-193a-5p	miR-193a-5p	3.979	0.015
	hsa-miR-152-3p	miR-148-3p/152-3p	17.618	0.001	hsa-miR-511-5p	miR-511-5p	4.827	0.003
	hsa-miR-193a-5p	miR-193a-5p	17.515	0.001	hsa-miR-652-3p	miR-652-3p	no expr. In no-AKI	0.028
	hsa-miR-27b-3p	miR-27-3p	18.184	0.001	hsa-miR-888-5p	miR-885-5p	6.600	0.019
	hsa-miR-320a	miR-320	-1.012	0.039				

Table 4

Table 4 – Pathway analysis summary as determined by the Qiagen Ingenuity software. Canonical pathways are variety of signalling or metabolic pathways available in the Ingenuity database. Renal pathways indicate nephrotoxic effect of certain molecules as listed in the Ingenuity database. Significance level (*p*-value) is calculated by Fisher's exact test and indicates the probability of association of molecules regulated by the identified miRNAs with above mentioned pathways by random chance alone. Pathway overlap indicates overlap of the identified miRNA-regulated molecules with the Ingenuity database. Target genes show genes affected by the identified miRNAs also present among the pathway's molecules. Bold target genes indicate experimentally verified miRNA targets.

Time point	Age group	Pathway		*p*-value	Pathway overlap	Target genes
Before surgery	All age groups	Canonical	2-amino-3-carboxymuconate Semialdehyde Degradation to Glutaryl-CoA	1.28E-02	10.00%	ACMSD
			dTMP De Novo Biosynthesis	1.79E-02	7.10%	**DHFR**
			Mitochondrial Dysfunction	2.43E-02	1.10%	PRDX3, VDAC1
			NAD Phosphorylation and Dephosphorylation	2.55E-02	5.00%	ACPP
			mTOR Signaling	2.86E-02	1.00%	EIF3M, RICTOR
		Renal	Renal Inflammation	1.79E-02 - 1.79E-02		**DHFR**
			Renal Nephritis	1.79E-02 - 1.79E-02		**DHFR**
			Renal Necrosis/Cell Death	1.10E-01 - 5.92E-02		PRDX3, VDAC1
			Renal Proliferation	2.47E-01 - 2.47E-01		VDAC1
	Children	Canonical	Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	6.39E-05	12.50%	CASP2*, HLA-A, HLA-B, HLA-C
			Neuroprotective Role of THOP1 in Alzheimer's Disease	2.69E-04	8.70%	PRKAR2A, HLA-A, HLA-B, HLA-C
			Colorectal Cancer Metastasis Signaling	3.41E-04	3.20%	ADCY10, **CASP3**, HNF1A, MMP10, PRKAR2A, PTGER3, PTGER4, **TGFBR1,**
			Factors Promoting Cardiogenesis in Vertebrates	4.17E-04	5.40%	HNF1A, NOG, PRKCZ, **TGFBR1**, TGFBR3

			Th2 Pathway	5.75E-04	4.00%	HLA-A, HLA-B, MAF, SOCS3, **TGFBR1**, TGFBR3
		Renal	Glomerular Injury	3.27E-01 - 4.54E-04		LAMB2, CHRNA7, **CASP3**, HNF1A, **MAFB**, **DICER1**
			Renal Proliferation	3.93E-03 - 3.93E-03		ATG5, BICC1, FGF1, PRKCZ, PTGER3, SOCS3
			Nephrosis	6.80E-03 - 6.80E-03		LAMB2
			Renal Dysfunction	6.80E-03 - 6.80E-03		HNF1A
			Renal Dysplasia	2.03E-02 - 8.06E-03		BICC1, ITGA8
	Infants	Canonical	Factors Promoting Cardiogenesis in Vertebrates	3.28E-05	7.60%	NOG, PRKCZ, **SMAD4, SMAD5**, SMAD9, **TGFBR1,** TGFBR3
			Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.52E-04	4.50%	APOC4, ARG2, MAP3K11, MAP3K13, **PPP1R7**, PPP2CA, PPP2CB, PRKCZ, SAA4
			PTEN Signaling	1.86E-04	5.80%	APOC4, ARG2, MAP3K11, MAP3K13, **PPP1R7**, PPP2CA, PPP2CB, PRKCZ, SAA4
			HIPPO signaling	2.09E-04	6.90%	**PPP1R7**, PPP2CA, PPP2CB, PRKCZ, **SMAD4, SMAD5**
			Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	2.54E-04	12.50%	CASP2*, HLA-A, HLA-B, HLA-C
		Renal	Renal Necrosis/Cell Death	2.47E-01 - 9.46E-05		ADORA1, **BAX**, BCL2L11, **CASP3**, DUSP, **F2**, FGF1, **GRB10**, **IGF1R**, **MAFB**, **PMAIP1**, RFXANK, **SGPL1**, TNFSF10, **VEGFA**, KIRREL, PTH1R
			Glomerular Injury	3.37E-01 - 2.81E-04		FOXC2, **VEGFA**, KIRREL, LAMB2, **MAFB**, BCL2L11, CHRNA7, **CASP3**, DUSP1, **DICER1**, **SMAD4**
			Nephrosis	9.83E-02 - 9.74E-03		LAMB2, ADORA1, PDE7B

			Renal Inflammation	4.78E-02 - 9.74E-03		ADORA1,**BAX**,BCL2L11,LAMB2,PDE7B,**PIGR**,PPP2CA,**SMAD4**,**VEGFA**
			Renal Nephritis	4.78E-02 - 9.74E-03		ADORA1,**BAX**,BCL2L11,LAMB2,PDE7B,**PIGR**,PPP2CA,**SMAD4**,**VEGFA**
After surgery	All age groups	Canonical	PTEN Signaling	2.34E-02	1.70%	BCL2L11, PDGFRB
			Arginine Degradation I (Arginase Pathway)	2.51E-02	7.70%	ARG2
			Arginine Degradation VI (Arginase 2 Pathway)	3.09E-02	6.20%	ARG2
			Urea Cycle	3.84E-02	5.00%	ARG2
			Citrulline Biosynthesis	4.97E-02	3.80%	ARG2
		Renal	Glomerular Injury	2.87E-01 - 5.86E-03		BCL2L11, PDGFRB
			Renal Necrosis/Cell Death	1.85E-01 - 8.60E-03		AMER1, BCL2L11, STRA6, TMX1, PTGR1
			Renal Proliferation	9.34E-02 - 9.34E-02		PDGFRB
			Renal Inflammation	1.27E-01 - 1.27E-01		BCL2L11
			Renal Nephritis	1.27E-01 - 1.27E-01		BCL2L11
	Children	Canonical	Dopamine Receptor Signaling	5.45E-04	5.50%	**PPP1R7**, PRKAR2A, PRL, PTH, PTS
			Antiproliferative Role of TOB in T Cell Signaling	8.75E-04	11.50%	CDC34, **SMAD4**, **TGFBR1**
			PTEN Signaling	1.95E-03	4.10%	**CASP3**, CSNK2A1, **IGF1R**, **TGFBR1**, TGFBR3
			STAT3 Pathway	2.11E-03	5.40%	**IGF1R**, MAP3K11, **TGFBR1**, TGFBR3
			Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	3.46E-03	3.00%	APOC4, ARG2, MAP3K11, MAP3K13, **PPP1R7**, SAA4
		Renal	Renal Atrophy	7.74E-02 - 2.58E-05		**ADAMTS1**,LRRK2,OVOL1,PTH,SLC4A1

			Renal Necrosis/Cell Death	4.83E-01 - 3.18E-03		ALDH3B1,**CASP3**,**F2**,**GRB10**,**IGF1R**,**MAFB**, PSIP1,RFXANK,**SGPL1**,TNFSF10, LRRK2
			Renal Inflammation	3.89E-01 - 7.29E-03		LRRK2,**PIGR**,SLC19A3,**SMAD4**
			Renal Nephritis	3.89E-01 - 7.29E-03		LRRK2,**PIGR**,SLC19A3,**SMAD4**
			Glomerular Injury	3.58E-01 - 1.45E-02		CHRNA7, **DICER1**, **SMAD4**, **MAFB**, **CASP3**
	Infants	Canonical	PTEN Signaling	5.52E-07	7.40%	BCL2, BCL2L11, **CASP3**, **FGFR1**, **IGF1R**, **MAPK3**, **RAF1**, **TGFBR1**, TGFBR3
			STAT3 Pathway	2.07E-06	9.50%	BCL2, **FGFR1**, **IGF1R**, MAP3K11, **RAF1**, **TGFBR1,** TGFBR3
			Ovarian Cancer Signaling	2.28E-05	5.50%	BCL2, **FGFR1**, **MAPK3**, PRKAR2A, **RAF1**, **VEGFA,** WNT16, WNT2B
			Apoptosis Signaling	7.87E-05	6.70%	AIFM1, BCL2, BCL2L11, **CASP3**, **MAPK3**, **RAF1**
			Melanocyte Development and Pigmentation Signaling	1.27E-04	6.20%	BCL2, **FGFR1**, **MAPK3**, PRKAR2A, **RAF1**, RPS6KA3
		Renal	Glomerular Injury	3.76E-01 - 5.11E-06		**ARHGDIA**,BCL2,CHRNA7,UMOD,**VEGFA**, **WT1**,CHRNA7,**DICER1**,**FGF2**,CDKN1C, CASP1
			Renal Necrosis/Cell Death	1.88E-01 - 5.05E-04		AMER1,BCL2,BCL2L11,**CASP3**,**F2**,**FGF2**,**GRB10**,**IGF1R**,**RAF1**,TNFSF10,UMOD,**VEGFA**,**WT1**
			Renal Inflammation	2.56E-01 - 6.20E-04		**ARHGDIA**,BCL2,BCL2L11,,TNFSF13B,UMOD,**VEGFA**,**WT1**
			Renal Nephritis	2.56E-01 - 6.20E-04		**ARHGDIA**,BCL2,BCL2L11,,TNFSF13B,UMOD,**VEGFA**,**WT1**
			Renal Damage	1.41E-01 - 1.13E-03		**ARHGDIA**, CHRNA7, **DICER1**, **FGF2**

Supplemental Digital Content

Methods

Study population

Study population included 24 consecutive patients who underwent surgery for congenital heart diseases at the East Midlands Congenital Heart Centre (Glenfield Hospital, Leicester, UK) between October 2014 and February 2015. Participants were prospectively recruited for this observational feasibility study if they were younger than 18 years old, their body weight was greater than 2 kg and their operation required cardiopulmonary bypass (CPB). Patients were excluded if they had a diagnosed pre-existing inflammatory state such as sepsis undergoing treatment or a chronic inflammatory disease and a diagnosis of acute kidney injury within 5 days before surgery. Emergency or salvage procedures were excluded, as well as patients supported by Extracorporeal Membrane Oxygenation (ECMO) or likely to require postoperative ECMO. Previous nephrotoxin use such as contrast solutions was not an exclusion factor as long as the serum creatinine (SCr) values were within reference range at the time of surgery. The study was approved by The East Midlands – Nottingham 1 Research Ethics Committee. Written informed consent from parents or legal guardians was obtained before enrolment and was confirmed before every invasive procedure when possible. Written informed assent was collected for patients aged 11 to 15 years where appropriate. Competent patients aged 16 or 17 provided their own consent.

Patients were defined as newborns if 30 days old or younger, infants if aged between 30 days and 12 months and children if older than 12 months.

All eligible patients received the standard preoperative care, as determined by the attending physician. All patients underwent surgery through a median sternotomy and the use of extracorporeal circulation. Anaesthesia was standardized where possible and was induced by servoflurane or isoflurane, along with the administration of neuromuscular blockade (rocuronium or

atracurium) and analgesia using fentanyl and/or morphine. Patients for whom servoflurane was unsuitable were given an intravenous induction using ketamine, propofol or midazolam. The cardiopulmonary bypass (CPB) was conducted with a non-heparin coated 'semi-closed circuit' (Maquet-Jostra, Hirrlingen, Germany) with a soft shell venous reservoir, a roller pump (Stockert, Munchen Germany), a microporous hollow fibre oxygenator (Quadrox, Maquet-Jostra, Hirrlingen, Germany) and a 40 micron arterial filter. The circuit was primed with a combination of the 10% mannitol, 8.4% sodium bicarbonate, plasmalyte pH 7.4, and succelylinated volplex. Temperature during CPB was managed after discussion with the surgical team and according to the operative technique. A red cell blood prime was used to maintain a haematocrit of > 30 g/L according to patient characteristics. All patients were admitted to the paediatric intensive care unit (PICU) following surgery. The use of inotropes, vasopressors or diuretics was at the discretion of the attending physician.

Feasibility outcomes

Recruitment and dropout rates were monitored. Protocol adherence was evaluated through the monitoring of protocol violations defined as any change, deviation, or departure from the study design or procedures of research project that was not approved prior to study initiation or implementation. Major violations were considered in case of a serious and/or continuous failure to comply with the protocol, standard operating procedures, good clinical practice (GCP) or trial regulations, if there was a significant negative impact to subject safety or if there was a significant damage to the completeness, accuracy and reliability of the data collected for the study. Minor protocol violations were considered if the previous definition was not applicable and included the failure to obtain specified clinical data, blood volume or urine samples required for analysis at the required time (deviation of more than 60 mins from the planned collection time). For an individual participant, the end of the trial was defined as 7 days after the index surgical procedure or as

hospital discharge if earlier. Laboratory protocol non-adherence was defined as the failure to complete laboratory essays following the study protocol.

Definition of AKI and clinical outcomes

The reference standard to classify AKI was defined according to the Kidney Disease: Improving Global Outcomes (KDIGO) criteria [1] as an increase in SCr by ≥ 0.3 mg/dl (≥ 26.5 µmol/l) or an increase in SCr to ≥ 1.5 times baseline, or a urine volume < 0.5 ml/kg/h for 6 hours. AKI was also staged according to the KDIGO guidelines. The time frame for the assessment of AKI was up to 7 days or hospital discharge if earlier. Urine output was routinely monitored until the end of the follow up period. Despite the uncertainty in defining AKI in neonates [2], KDIGO criteria were applied to the whole population in the absence of specific criteria for new-borns in guidelines. In addition, to reflect the fact that young infants may be unable to generate high serum creatinine values we also used estimated creatinine clearance was used to define Stage 3 AKI, using the Schwartz equation,[3] as per the pRIFLE criteria.

Preoperative data likely to influence the post-operative recovery and operative data such as procedure details, bypass time and cross clamp time were recorded in a prospective ad hoc database. Risk adjustment for congenital heart surgery (RACHS-1) category was assigned to each surgical procedure performed [4]. Postoperative morbidity and mortality, duration of mechanical ventilation, ICU and hospital stay and other non-biochemical outcomes were collected up to seven days after surgery or discharge if earlier. The Pediatric Multiple Organ Dysfunction Score (P-MODS) [5] and the Vasoactive-Inotropic Score (VIS) [6] were monitored during PICU stay. Post-operative gas analysis and routine blood results were carried out according to the attending physician's decision and were recorded until seven days after surgery or discharge if earlier.

Biochemical markers

Serum and urine concentrations of creatinine (UCr and SCr, respectively) were determined using creatinine assay kit (Abcam, Cambridge UK) as recommended by the manufacturer Concentration of troponin cTnI was estimated in serum using a commercial kit from Enzo Life sciences (Lausanne, Switzerland). Urine neutrophil gelatinase-associated lipocalin (NGAL) was measured in urine using ELISA kit (EKF Diagnostics, Cardiff UK).

Concentrations of interleukin-6 (IL-6), interleukin-8 (IL-8), tumour necrosis factor α (TNFα), monocyte chemoattractant protein 1 (MCP-1), monocyte chemoattractant protein 3 (MCP-3) and macrophage inflammatory protein 1 (MIP-1), as well as markers of endothelial injury such as circulating intercellular adhesion molecule (ICAM) and E-selectin were simultaneously quantified in plasma using the ProcartaPlex™ immunoassay kits (eBioscience, San Diego, CA, USA) and measured with the MagPix multiplex array system employing Luminex xMAP technology in combination with the xPonent Software (Luminex corporation, Austin Texas US).

Haemoglobin concentration was calculated as previously described [7]. Briefly, the samples were spun at 1,800 xg for 15 min and the resulting supernatant was further spun for 5 min at 20,000 xg. The supernatant was diluted 5x with PBS and absorbance measured at 415nm, 450nm and 700nm using Multiplate reader Enspire (PerkinElmer). Haemoglobin concentration was calculated as follows: Hb = 1.58xA415 - 0.95xA450 - 2.91xA700.

Platelet and leukocyte activation was estimated by flow cytometry (Cyan ADP, Beckman Coulter, Brea, CA) in citrated patients' blood samples. For platelets samples were labelled with FITC-coupled CD62E (Abcam, Cambridge, UK), and PE-coupled CD41, Affymetrix, Santa Clara, CA, USA). For leucocyte FITC-labelled CD64 and PE-labelled CD163 (Affymetrix) were used and red cells were lysed using FACSlyse solution from BD Biosciences.

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Supplemental Figure legend

Supplemental Figure 1 – Study design and kidney function A – STROBE diagram, **B** – Serum creatinine. Values in line plots represent means ±SD.

Supplemental Table 1

Supplemental Table 2

Supplemental Table 3

Supplemental Table 4

Supplemental Table 5