DETECTION OF INTRAVASCULAR HEMOLYSIS IN NEWBORNS USING URINARY CARBONIC ANHYDRASE I IMMUNOREACTIVITY

Alzbeta Hulikova[1], Holger Kramer[1,3], Hammad Khan[2#], Pawel Swietach[1]*

[1] *Department of Physiology, Anatomy & Genetics, University of Oxford, Parks Road, Oxford OX1 3PT, UK*

[2] *Evelina London Children's Hospital, Neonatal Unit 6th Floor, North Wing, St Thomas' Hospital, Westminster Bridge Road, London SE1 7EH, UK*

[3] *Current address: MRC London Institute of Medical Sciences, Du Cane Road, London W12 0NN, UK*

Running head: *Urinary CAI as a biomarker of neonatal hemolysis*

*Address for correspondence:
Dr Pawel Swietach, Department of Physiology, Anatomy & Genetics, University of Oxford, Parks Road, Oxford OX1 3PT, England, Email: pawel.swietach@dpag.ox.ac.uk,

Human genes: *Car1*

Trial registration: CPMS 19576.

ABSTRACT

BACKGROUND: Mild hemolysis occurs physiologically in neonates, but more severe forms can lead to life-threatening anemia. Newborns in developing regions are particularly at-risk due to the higher incidence of triggers (protozoan infections, sepsis, certain genetic traits). In advanced healthcare facilities, hemolysis is monitored indirectly using resource-intensive methods that probe downstream ramifications. These approaches could potentially delay critical decisions in early-life care, and are not suitable for point-of-care testing. Rapid and cost-effective testing could be based on detecting red blood cell (RBC)-specific proteins, such as carbonic anhydrase I (CAI), in accessible fluids (e.g. urine).

METHODS: Urine was collected from twenty-six full-term male neonates and analyzed for CAI using immunoassays (ELISA, western blot) and proteomics (mass spectrometry). The cohort included a range of hemolytic states, including admissions with infection, ABO incompatibility, and receiving phototherapy. Data were paired with hemoglobin, serum bilirubin (SBR) and C-reactive protein (CRP) measurements.

RESULTS: Urine from a control cohort (CRP<20 mg/L, SBR<125μmol/L) had no detectable CAI, in line with results from healthy adults. CAI excretion was elevated in neonates with raised SBR (>125 μmol/L), including those qualifying for phototherapy. Newborns with low SBR (<125 μmol/L) but elevated CRP (>20 mg/L) produced urine with strong CAI immunoreactivity. Proteomics showed that CAI was the most abundant RBC-specific protein in CAI-immunopositive samples, and did not associate with other RBC-derived peptides, indicating an intravascular hemolytic source, followed by CAI-selective excretion.

CONCLUSIONS: CAI is a direct biomarker of intravascular hemolysis that can be measured routinely in urine using non-invasive methods under minimal-laboratory conditions.

IMPACT STATEMENT

Hemolysis in the newborn can be life-threatening, yet direct assays for rapid diagnosis are not available for regular monitoring. Instead, current clinical management relies on resource-intensive measurements of downstream ramifications, potentially delaying critical decisions in early-life care. Using a cohort of newborns manifesting various hemolytic states, we show that intravascular hemolysis can be detected by measuring CAI excretion in a small sample of urine using cost-effective immunoreactivity techniques. Since this biomarker reports cell-rupture, it provides a more direct readout of hemolysis. The method can improve resource allocation, identify 'at-risk' patients earlier, and be implemented under minimal-laboratory conditions.

INTRODUCTION

Hemolysis is the rupturing of red blood cells (RBCs) that results in the release of their cytoplasmic contents. Various degrees of intravascular hemolysis take place in the first days of life. A mild, physiological form of hemolysis facilitates the process of replacing fetal hemoglobin with its adult form *(1)*. More severe forms, manifesting as jaundice, are associated with medical conditions, such as sepsis (e.g. Gram-positive bacteria), protozoan infection (e.g. malaria), alloimmunity (ABO and Rhesus incompatibility), genetic traits (e.g. sickle cell, glucose-6-phosphate dehydrogenase deficiency [G6PDD]), birth trauma and prematurity. Severe neonatal jaundice represents a significant burden of morbidity worldwide, and is particularly problematic in developing regions, where hemolytic triggers are more prevalent *(2-4)*. Further, the incidence of certain hemolytic conditions (e.g. sickle cell trait, Rhesus disease, G6PDD) is related to ethnicity, resulting in at-risk groups *(5-8)*.

Since hemolysis can lead to life-threatening anemia, successful management must rely on early and accurate diagnosis. The timing, duration and intensity of a hemolytic crisis cannot be readily predicted, and therefore at-risk patients should be monitored regularly, using assays that provide a direct, rapid and linear readout of RBC rupture. Certain causes of hemolysis can be diagnosed with specific tests, such as Coombs test for autoimmune hemolytic anemia *(9)*, but these are resource-intensive and are unable to quantify the degree of ongoing hemolysis. The standard clinical tests for hemolysis involve taking blood for cell count, morphology or biochemical assays (e.g. for unconjugated bilirubin, haptoglobin).

Serum haptoglobin is commonly used as an inverse biochemical assay of hemolysis (i.e. levels fall during hemolysis), but its interpretation can be ambiguous if the baseline of circulating haptoglobin is not established with adequate precision. A linear and non-saturating positive marker, such as serum bilirubin (SBR), overcomes this issue. However, this and

similar diagnostic approaches do not provide a direct readout of hemolysis, in the sense that they do not probe for RBC-specific molecules released upon rupture. Instead, these interrogate the downstream cascades, which inadvertently introduces a delay in diagnosis. Moreover, babies diagnosed with jaundice on the basis of bilirubin measurements are often assumed to be hemolysing, although a small proportion may have a severe non-hemolytic condition, such as Crigler–Najjar syndrome which requires urgent medical attention. Identification of such cases would be expedited by a direct assay for hemolysis. Whilst blood-based tests are performed routinely in hospitals, they require trained personnel, laboratory equipment and high standards of hygiene, and thus may not be feasible for ambulatory care, in developing countries and outside advanced healthcare systems. These onerous requirements also preclude high-frequency, point-of-care testing in at-risk groups to accurately track hemolysis.

Given that neonatal hemolysis is a global health concern, there is a medical and economic incentive for developing RBC-related biomarker tests that are fast, reliable, quantitative and safe, yet technically simple and low-cost. Proteins released from ruptured RBCs are good candidate-molecules for hemolysis biomarkers, provided they are *(i)* stable, *(ii)* distinguishable from potential non-RBC sources and *(iii)* released in sufficient amount to produce a detectable signal. A desirable property of such a biomarker would be detection in readily accessible body fluids, such as urine, rather than in blood, for repeated testing.

Carbonic anhydrase I (CAI) is a soluble protein that is highly abundant in the cytoplasm of RBCs *(10, 11)* (~4 g/L of adult blood, ~1 g/L of neonatal blood *(12, 13)* and rising over the first year of life *(14)*), but not highly expressed in other tissues, where levels are typically orders of magnitude lower than inside red cells *(15, 16)*. The size of the CAI pool in RBCs would be sufficient to raise plasma CAI upon hemolysis, even after accounting for volume-dilution. Normally, only trace quantities of CAI are found in plasma (~0.001 g/L) *(13, 17)* thus providing very low background. Given its small size (29 kDa, Stokes radius 25 Ang) *(12, 15, 18)*, CAI

is expected to cross the glomerular barrier and appear in urine, as has been suggested by a small-scale study of adults with renal disease *(19)*. However, the relationship between hemolysis and urinary CAI excretion is unknown. After glomerular filtration, CAI protein may, for example, be reclaimed by megalin/cubilin receptors in the kidney proximal tubule fluid *(20)*. One previous report has detected only trace amounts of CAI in the urine of apparently healthy adults (<5 μg/day) *(17)*, but not explored its relationship with hemolysis. It has not yet been determined if CAI is detectable in the urine of hemolysing neonates, and whether this information carries any diagnostic meaning.

Here, we assayed urinary CAI excretion in neonates that included non-hemolysing controls and various conditions expected to produce a spectrum of hemolytic states. We show that the urinary CAI signal ranges from undetectable to strong, and relates to the underlying hemolytic condition. By adapting this method for lateral-flow immunochromatography 'strip-tests', it would be possible to obtain rapid readouts of hemolysis for point-of-care testing in the newborn.

METHODS

Patients. Patient recruitment took place at Evelina London Children's Hospital over a period of 12 months (starting August 2015), where admissions include prematurity, respiratory distress, sepsis, as wells as cardiac, neurological and surgical conditions (~800 cases per year). For this study, 26 full-term babies were recruited; none were excluded. The cohort included babies with jaundice requiring treatment, mildly jaundiced, and those with no hemolytic condition (controls). In order to obtain a sufficient volume of urine for multiple types of analyses, male babies were recruited. Urine samples were obtained in the first 9 days of life

and then anonymized. For this study, urine bags were used to eliminate possible fecal contamination of samples. Clinical information (date of birth, gestation, phototherapy, treatment with antibiotics) and standard clinical measurements of (i) blood hemoglobin, (ii) serum bilirubin, (iii) plasma [K^+], (iv) plasma [creatinine], (v) C-reactive protein were taken but not made available to the person collecting or measuring from urine samples. Urine samples were frozen for storage (−20 deg C) in 1.5 mL labelled tubes pre-treated with 100 µL of protease inhibitor solution (Complete, Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics; 1 tablet/10 mL of final volume), and thawed when ready for measurements. To test the methodology with negative samples, urine was taken from a cohort of non-hemolysing healthy adult volunteers (University of Oxford Central University Research Ethics Committee procedure 24). See supplement for power calculations.

Measurements on urine samples. Urine samples were centrifuged to remove solid deposits and aliquoted to provide at least 200 µL for immunotechniques (ELISA, immunoblots) and measurements of protein and creatinine. See Supplement for details. In the case of neonatal urine, 200-500 µL was reserved for mass spectrometry, which included a digestion step and measurement of total peptide. Any residual neonatal urine was destroyed before the end of the study period. Any residual urine remaining from adults was destroyed within 24 hours of collection.

ELISA. Each urine sample was measured in triplicate. 50 µl aliquots of urine were added to each well of a 96-well high-binding microplate (Greiner Bio-One). As a positive control, lysates of RBCs were dissolved in adult urine. The plate was then incubated overnight at 37°C and air-dried, washed with 0.05% phosphate-buffered saline (PBS) with Tween detergent (PBST), blocked with 10% fetal calf serum (FCS) in PBS for 2 hours, and incubated with primary goat anti-human CAI polyclonal antibody (R&D Biosystems, Novus) diluted 1:250 in blocking solution (1hr at room temperature). Next, the plate was washed four times

with PBST, and incubated with HRP-conjugated rabbit anti-goat IgG (H+L) secondary antibody, diluted 1:8000 in blocking buffer (1hr at room temperature), and then washed again. The signal was developed using OPD solution (Sigma/Merck) and absorbance was measured at 490nm (Biotek ELx800 spectrophotometer or Cytation 5, Biotek). Background absorbance was measured in wells that contained PBS.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). See supplement for details of protocol *(21)* and analysis (MaxQuant v1.5.8.3) *(22)*. Data were searched against a UniProt human database (v20170202).

Statistics. Significance of ELISA signal relative to background signal was tested by two-tail t-test. The relationship between urinary CAI status and clinical information was tested by the Freeman-Halton extension of Fisher's exact probability test and Chi-squared test of a 2-by-3 contingency table. Significance of differences in the abundance of urinary proteins was tested by one-way ANOVA.

Study approval. This prospective study was given ethical approval by the UK National Health Service Research Ethics Committee NRES (REC reference: 14/NS/0042, IRAS project ID: 170697). Written consent was obtained from the parent/legal guardian of the recruited newborn, or from adult donors themselves.

RESULTS

RBC-derived CAI produces a strong signal for detection by immunoreactivity assays

The antibody against CAI was tested for cross-reactivity with CAII, a related isoform,

purified from bovine blood. Strong immunoreactivity was detected in lysates prepared from human RBCs, but not with purified CAII, confirming isoform-specificity (**Figure 1A-B**). Human RBC-derived CAI could be detected in dilutions up to 10^5-fold in urine using ELISA (**Figure 1C**). For this experiment, background absorbance was determined in wells containing PBS instead of urine. Urine samples from adult volunteers (N=8; mean age: 34 years) showed no detectable CAI by ELISA (**Figure 1D**), as expected from a minimal degree of hemolysis and confirming previous studies of only trace CAI excretion. This negative result was confirmed by western blotting, a more sensitive method (**Figure 1E**; note, prolonged film exposure revealed only trace CAI immunoreactivity in some samples; **Figure 1F**).

The linearity of CAI ELISA was demonstrated using various dilutions of human recombinant CAI (hrCAI) in adult urine (**Figure S1**). This calibration curve was used in subsequent analyses to calculate the amount of CAI (in ng/well). To test the assay's reporting accuracy, a spike/recovery study was performed on urine samples from 4 adults (**Figure S2**). Samples were split into two groups, one of which was enriched in filtered (<100 kDa) hemolysate, prepared from human venous blood. Spiking was performed with known amounts of hrCAI. Absorbance measurements, performed before and after spiking (**Figure S2A**), were converted to an amount of CAI, which was then expressed relative to the known amount of hrCAI added (**Figure S2B**). The recovery for all spikes tested was close to 100%, confirming the assay's accuracy. To determine the assay's precision, repeated measurements on hrCAI-spiked adult urine samples were performed over a period of 8 days (**Figure S3**). The ELISA signal remained consistent, indicating a good level of precision.

Urine samples from newborn manifest a range of CAI immunoreactivity

Urine was obtained from 26 full-term babies (gestation time: 37.0-42.4 weeks; mean

39.4 weeks) (**Figure 2A**), collected between the 2nd and 9th day of life (mode 3 days) (**Figure 2B**). Blood hemoglobin (Hb) ranged from 107 to 245 g/L (mean 174 g/L, SD 30 g/L); twenty-two babies had Hb in the normal range (134-199 g/L), two had Hb<134 g/L and two had Hb>199 g/L (**Figure 2C**). Plasma [K^+] was 3.6-6.6 mM (mean 5.1 mM, SD 0.84 mM), with two samples classified as hyperkalemic (>6.0 mM). Mean plasma creatinine, urine creatinine, and the urine/plasma creatinine ratio were 54 µmol/L (SD 22 µmol/L), 25 µmol/L (SD 18 µmol/L) and 0.47 (SD 0.27), respectively. Renal function was normal (urinary protein/creatinine<1.5 g/g) in all, but two patients *(23)* (**Figure 2D**).

CAI immunoreactivity in neonatal urine was tested by ELISA. Background signal was determined in wells that contained PBS. Unlike in the case of healthy adults, where CAI levels in urine were consistently negligible (**Figure 1D**), neonates produced urine with a wide range of CAI immunoreactivity determined by ELISA (**Figure 2E**). This absorbance readout was converted into an amount of CAI using the calibration curve shown in **Figure S1**. Signals ranged from nil (i.e. at background level) in 9 samples, to a spectrum of positive signals in the remaining 17 samples, the strongest of which was equivalent to 15 ng CAI per well (50 µL). CAI status was confirmed by western blot (**Figure 2F**). These findings provide the first evidence that significant levels of CAI-specific immunoreactivity can be measured in neonatal urine.

The use of urine bags on male babies in this trial eliminates the possibility of fecal contamination. However, such contamination cannot be excluded if more crude methods are attempted to collect urine. Previous studies have demonstrated CAI expression in colorectal epithelium *(16, 24)*, and this could potentially influence CAI readouts in urine contaminated with feces. To test if the presence of colorectal epithelium could meaningfully compromise the robustness of CAI measurements, ELISA measurements were performed on adult urine samples containing either filtered hemolysate (750x-diluted) or lysates prepared from human

colorectal cell lines (Caco2, DLD1, HCT116 and HT29) (**Figure S4A**). Total protein concentration was measured to derive the CAI content per unit of total protein. A CAI signal equivalent to 1 ng required only ~0.1 μg of hemolysate, but as much as 500 μg of colorectal epithelium lysate (**Figure S4B**). This difference is sufficiently large to indicate that fecal contamination is unlikely to influence urinary CAI levels. To confirm this experimentally, ELISA measurements were performed on adult urine dosed with human feces. Contaminated urine was produced by adding a sample of feces to urine (1:10 v/v), followed by vigorous mixing and two rounds of ultracentrifugation (9000 rpm, 10 min, 4°C). As a positive control, urine was spiked with hrCAI. As shown in **Figure S5**, fecal contamination as high as 10% did not affect background CAI signal, indicating that feces are highly unlikely to produce a false-positive immunoreactivity. Thus, any urinary CAI excretion would be attributable to hemolysis.

Urinary CAI positivity is associated with increased levels of bilirubin and C-reactive protein

To investigate the relationship between urinary CAI excretion and clinical presentation, data were stratified according to phototherapy status. Twelve neonates receiving phototherapy had raised serum bilirubin (SBR), with a mean of 267 μmol/L (SD 44 μmol/L), on the day of urine collection. Among those not qualifying for phototherapy, three neonates had elevated C-reactive peptide (CRP>20 mg/L) *(25)* and received antibiotics, indicating an underlying infection. The remaining neonates (11) manifested a range of SBR levels, from low to elevated, and were further subdivided into two groups, taking an SBR cut-off of 125 μmol/L. The cohort with low SBR (<125 μmol/L) was the 'control' group, and that with raised SBR (>125 μmol/L) but not meeting the criteria for phototherapy was referred to as the 'subclinical' group (**Figure 3A**).

The relationship between SBR and urinary CAI signal (ELISA) delineates these four groups (**Figure 3B**). Absorbance was significantly above background (cut-off 0.05075 as measured in PBS) in the 'infected', 'phototherapy' and 'subclinical' groups (**Figure 3C**). The 'infected' group had the highest CAI signals (3/3 CAI-immunopositive), but presented no evidence for renal failure (**Figure 2D**), which argues for infection-related hemolysis. Strikingly, ten of the 12 neonates receiving phototherapy, including two with a positive direct Coombs test, produced CAI-immunopositive urine. In contrast, none of the neonates in the control group excreted CAI in their urine. Differences in CAI immunoreactivity between the four groups were not explained by age at which urine was sampled (**Figure 3D**; P=0.62, one-way ANOVA).

An association between urinary CAI-immunopositivity (a direct product of hemolysis) and elevated SBR (a downstream readout of hemolysis) was tested using a 2x3 contingency table for 'control', 'subclinical' and 'phototherapy' groups, representing increasing levels of SBR (mean±SD: 75±36 μmol/L, 173±35 μmol/L, 267±44 μmol/L). Fisher's exact probability test and Chi-squared test indicated a significant association between CAI status and SBR (P=0.005; **Table 1**).

The protein signature of urine indicates an intravascular hemolytic source of urinary CAI

The detection of a CAI signal in urine implicates a large source of CAI, namely a population of ruptured RBCs, as no other tissue would be capable of releasing a sufficient amount of CAI. However, the results thus far cannot determine whether RBC rupture had taken place inside blood vessels or along the urinary tract. An extravascular hemolytic event would result in the urinary excretion of RBC proteins that do not normally cross the glomerular filter (e.g. membrane-bound or large soluble proteins), or those proteins that are normally reclaimed

by the nephron. A related question is whether CAI is the most abundant RBC-specific protein detectable in urine. These two questions were addressed by proteomic analyses of urine.

Mass spectrometry analysis of neonatal urine identified over 1000 proteins in one or more of the 25 samples (**Table S1; Fig S6A**; note, there was insufficient volume in sample 27 to perform this analysis). To identify RBC-associated proteins, the urinary proteome was compared against a database of RBC proteins (**Fig S6B**) *(10)*. Among the 64 most abundant proteins in RBCs, collectively representing a third of all RBC peptides *(10)*, twenty-one were detected in at least one urine sample. Of these, CAI had the highest mean signal and showed greatest variation, i.e. potential dynamic range (**Figure 4A**). Importantly, ankyrin-1 (ANK1), hemoglobin A (HBA) and hemoglobin B (HBB), which are RBC-specific, were absent or appeared at very low levels in urine. Ankyrin-1 (206 kDa) is too large to cross the glomerular filter *(26)*, and any filtered hemoglobin would be reclaimed by the megalin/cubilin receptor system *(20)*, thus their absence from urine is consistent with an intravascular hemolytic event.

A comparison of the proteomes of CAI-immunopositive and CAI-immunonegative urine confirms that CAI peptides were significantly enriched in the former (**Figure 4B**). An analysis of urine by patient grouping confirmed CAI enrichment in the infected, phototherapy and subclinical groups (**Figure 4C;** one-way ANOVA, P=0.021). Phosphoglycerate kinase 1 was also differentially abundant in the four groups (P=0.008), but demonstrated lower intensity than CAI, making it a less suitable biomarker.

DISCUSSION

This proof-of-principle study demonstrates that CAI can be detected in neonatal urine

by immunoreactivity methods (**Figure 2**) and mass spectrometry (**Figure 4, Table S1**). In the cohort of full-term neonates, two-thirds produced distinctly CAI-immunopositive urine (**Figure 2B**). The remaining third of neonates produced urine with only trace CAI immunoreactivity, similar to that excreted by healthy adults (**Figure 1D**) and consistent with previous reports that CAI is not normally excreted in urine. The source of CAI in urine can be explained in terms of intravascular hemolysis of RBCs, the largest pool of CAI in the body. Moreover, urinary CAI excretion correlated with serum bilirubin, an independent but indirect indicator of hemolysis (**Figure 3**). Fecal contamination cannot account for urinary CAI immunoreactivity because measurements on feces-contaminated adult urine (**Figure S5**) and adult urine dosed with lysates of colorectal epithelial cells (**Figure S4**) produced negligible CAI signals.

Although the appearance of CAI in urine may be predicted from the properties of the glomerular filter *(26)*, urinary CAI excretion has not been demonstrated in hemolysing neonates. To explain the urinary CAI signal in immunopositive samples, CAI protein must have been released from a sizable pool of RBCs and then crossed the glomerulus, without subsequent degradation or reabsorption *(20)*. The highest urinary CAI immunoreactivity was equal to a 100-fold dilution of RBC lysate, and the mean signal among CAI-immunopositive samples was equivalent to a 10^5-fold dilution (**Figure 2E** *cf* **Figure 1C**). The appearance of CAI in urine samples collected for this study cannot be explained by an extravascular hemolysis along the urinary tract because CAI was not excreted with other RBC-specific proteins, such as ankyrin-1 and hemoglobin (**Figure 4B**). This lack of association between CAI and other RBC-specific proteins indicates that the hemolysate underwent filtration and selective reabsorption that eliminated ankyrin-1 and hemoglobin, but allowed CAI to pass.

Measurements of CAI in urine have excellent signal-to-noise ratio for detecting hemolytic events. The low background and positive correlation with hemolysis compare

favorably to the marker haptoglobin, which falls during hemolysis and therefore require accurate baseline information to perform the necessary subtraction. Also, CAI excretion is expected to increase proportionally with the degree of hemolysis, and therefore the detected signal is less prone to saturate. Under non-hemolysing conditions, urinary CAI signal is close to zero, which compares favorably to markers such as reticulocyte count for which there is always a non-zero baseline level in blood and therefore a narrower dynamic range. Markers such as LDH are not specific to ruptured RBCs, and can yield false-positives. CAI testing is more directly traceable to hemolysis, and therefore does not share the potential ambiguity of bilirubin measurements, which in rare cases such as Crigler–Najjar syndrome may lead to an erroneous diagnosis of hemolysis.

Urine samples with the highest CAI immunoreactivity were obtained from three neonates with significantly raised CRP and in receipt of antibiotics to treat an underlying infection (**Figure 2B**). Such CAI levels are indicative of a substantial degree of hemolysis. The source of CAI was not an extravascular hemolysis due to a urinary tract infection (UTI; a rare event in the first days of life *(27)*) because three major RBC-related proteins (ankyrin-1, hemoglobin alpha and beta) were detected at much lower intensities than CAI (400-, 15- and 100-fold, respectively). This discrepancy indicates that RBC proteins had been separated by the kidney. Furthermore, there was no proteomic evidence for markers of squamous epithelium nor neutrophil effector functions, which would be expected in a UTI *(28)*. The squamous epithelium markers cytokeratins and periplakins were not detected in any sample, and there was no significant difference in markers serpin B3, cornulin and desmoplakin between the high- and low-CRP cohorts (P=0.35, 0.89, and 0.17, respectively). Neurophil activity markers cathelicidins and calprotectins were not detected in any sample, and there was no significant difference in markers defesin-1, myeloperoxidase, cathepsin G, cathepsin L1 or pro-cathepsin H between the high- and low-CRP cohorts (P=0.92, 0.69, 0.72, 0.63, 0.57, respectively).

In accordance with routine clinical practice, based largely on SBR measurements, 12 of the recruited neonates were placed on phototherapy (National Institute for Health and Care Excellence guidelines), and among these, urinary CAI was detected in ten. The mean signal in this clinically jaundiced group was significantly above background, but not as high as in the group with elevated CRP, reflective of a less severe but still significant hemolytic event. Among those neonates that did not qualify for phototherapy, half manifested raised SBR and had a significantly elevated mean CAI signal. The remaining neonates, characterized by low SBR (<125 μmol/L), produced urine with no detectable CAI (**Figure 3C**). Data stratified by SBR level showed a significant association with CAI immunopositivity (**Table 1**), thus the presence of CAI in urine is meaningfully related with an established clinical index of hemolysis. Since the appearance of CAI in urine is expected to peak *before* the onset downstream biochemical changes, we propose urinary CAI excretion to be an early biomarker of hemolysis. To establish these dynamics, follow-up trials should track the time course of urinary CAI excretion in a larger cohort of newborns, and correlate these data with hemolytic markers, including serum haptoglobin.

The ELISA-based CAI detection method could be implemented in lateral-flow immunochromatography devices (strip-tests) for rapid readouts under minimal-laboratory conditions (i.e. similar to pregnancy kits). Testing could thus be performed regularly to track the onset and progression of hemolysis in early-life care with the necessary sampling frequency. This compares favorably to blood-based tests for LDH, haptoglobin or SBR, which cannot achieve such high temporal resolution. A detectable rise in CAI may precede other markers of hemolysis and thus improve resource allocation in the clinic and identify ‘at-risk’ patients earlier. This methodology may be particularly useful in economically-deprived regions with inadequate health care systems, in ambulatory care, and in developing countries where the incidence of hemolytic triggers (e.g. malaria, sickle cell disease) is higher.

AUTHOR CONTRIBUTIONS

A. Hulikova, experimental design, measurements and analysis; H. Khan, clinical lead and provision of samples and clinical data; H. Kramer, urine proteomics; P. Swietach, financial support, statistical analysis, administrative support, experimental design, analysis and wrote the paper.

ACKNOWLEDGEMENTS

We thank Drs Oliver Lomas and Killian Donovan (John Radcliffe, Oxford) for critically commenting on the manuscript. The authors have no conflicts of interest to declare. Research Funding: OUP-John Fell Fund (152/042). Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, preparation of manuscript, or final approval of manuscript.

REFERENCES

1. O'Brien RT, Pearson HA. Physiologic anemia of the newborn infant. J Pediatr 1971;79:132-8.

2. Murray NA, Roberts IA. Haemolytic disease of the newborn. Arch Dis Child Fetal Neonatal Ed 2007;92:F83-8.

3. Slusher TM, Zamora TG, Appiah D, Stanke JU, Strand MA, Lee BW, et al. Burden of severe neonatal jaundice: A systematic review and meta-analysis. BMJ Paediatr Open 2017;1:e000105.

4. Mitra S, Rennie J. Neonatal jaundice: Aetiology, diagnosis and treatment. Br J Hosp Med (Lond) 2017;78:699-704.
5. Dhaliwal G, Cornett PA, Tierney LM, Jr. Hemolytic anemia. American family physician 2004;69:2599-606.
6. Moise KJ. Hemolytic disease of the fetus and newborn. Clinical advances in hematology & oncology : H&O 2013;11:664-6.
7. Kumar S, Regan F. Management of pregnancies with rhd alloimmunisation. Bmj 2005;330:1255-8.
8. Moise KJ, Jr. Management of rhesus alloimmunization in pregnancy. Obstetrics and gynecology 2008;112:164-76.
9. Parker V, Tormey CA. The direct antiglobulin test: Indications, interpretation, and pitfalls. Arch Pathol Lab Med 2017;141:305-10.
10. Bryk AH, Wisniewski JR. Quantitative analysis of human red blood cell proteome. J Proteome Res 2017;16:2752-61.
11. D'Alessandro A, Righetti PG, Zolla L. The red blood cell proteome and interactome: An update. J Proteome Res 2010;9:144-63.
12. Maren TH. Carbonic anhydrase: Chemistry, physiology, and inhibition. Physiol Rev 1967;47:595-781.
13. Wahlstrand T, Knuuttila KG, Wistrand PJ. A radioimmunosorbent technique for the assay of b- and c-types of carbonic anhydrase in human tissues. Scand J Clin Lab Invest 1979;39:503-9.
14. Brady HJ, Sowden JC, Edwards M, Lowe N, Butterworth PH. Multiple gf-1 binding sites flank the erythroid specific transcription unit of the human carbonic anhydrase i gene. FEBS Lett 1989;257:451-6.

15. Sly WS, Hu PY. Human carbonic anhydrases and carbonic anhydrase deficiencies. Annu Rev Biochem 1995;64:375-401.
16. Tashian RE. The carbonic anhydrases: Widening perspectives on their evolution, expression and function. Bioessays 1989;10:186-92.
17. Backman U, Danielsson B, Wistrand PJ. The excretion of carbonic anhydrase isozymes ca i and ca ii in the urine of apparently healthy subjects and in patients with kidney disease. Scand J Clin Lab Invest 1990;50:627-33.
18. Supuran CT. Carbonic anhydrases: Novel therapeutic applications for inhibitors and activators. Nature reviews Drug discovery 2008;7:168-81.
19. Robinson JR. Urinary excretion of carbonic anhydrase: A simple test for the detection of intravascular haemolysis. Journal of clinical pathology 1950;3:142-5.
20. Christensen EI, Birn H. Megalin and cubilin: Multifunctional endocytic receptors. Nat Rev Mol Cell Biol 2002;3:256-66.
21. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods 2009;6:359-62.
22. Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed maxlfq. Mol Cell Proteomics 2014;13:2513-26.
23. El Hamel C, Chianea T, Thon S, Lepichoux A, Yardin C, Guigonis V. Normal values of urine total protein- and albumin-to-creatinine ratios in term newborns. Pediatr Nephrol 2017;32:113-8.
24. Sowden J, Leigh S, Talbot I, Delhanty J, Edwards Y. Expression from the proximal promoter of the carbonic anhydrase 1 gene as a marker for differentiation in colon epithelia. Differentiation 1993;53:67-74.

25. Perrone S, Lotti F, Longini M, Rossetti A, Bindi I, Bazzini F, et al. C reactive protein in healthy term newborns during the first 48 hours of life. Arch Dis Child Fetal Neonatal Ed 2018;103:F163-F6.
26. Chang RL, Deen WM, Robertson CR, Brenner BM. Permselectivity of the glomerular capillary wall: Iii. Restricted transport of polyanions. Kidney Int 1975;8:212-8.
27. Visser VE, Hall RT. Urine culture in the evaluation of suspected neonatal sepsis. J Pediatr 1979;94:635-8.
28. Yu Y, Sikorski P, Smith M, Bowman-Gholston C, Cacciabeve N, Nelson KE, Pieper R. Comprehensive metaproteomic analyses of urine in the presence and absence of neutrophil-associated inflammation in the urinary tract. Theranostics 2017;7:238-52.

TABLE 1: 2-by-3 contingency table for the Fisher Exact Probability Test. P=0.0052. Chi-squared test (χ^2=10.4, degrees of freedom=2), P=0.005.

Urine CAI status (by ELISA):	Not receiving phototherapy		Receiving phototherapy
	SBR<125 µmol/L	SBR>125 µmol/L	
CAI-immunonegative	5	2	2
CAI-immunopositive	0	4	10

FIGURE LEGENDS

FIGURE 1: *Testing CAI antibodies and immunoreactivity in adult urine.* **(A)** Specificity of the CAI antibody was tested by western blot on human RBC lysates diluted in urine (a source of CAI and CAII), and CAII purified from bovine blood. Dilutions were performed in urine from a non-hemolysing adult volunteer. **(B)** Comparing antigen recognition by anti-CAI and anti-CAII antibodies using dot blot (5ng bovine CAII per 2µL/dot and 2µL of 45-fold diluted human RBC lysate/dot). **(C)** RBC lysates, prepared from an adult volunteer, were diluted serially in urine. ELISA using the CAI antibody was performed and quantified in terms of absorbance. **(D)** CAI immunoreactivity measured by ELISA in urine samples from eight adult male volunteers. Line shows background signal, determined in wells containing PBS only. **(E)** Western blot of adult urine samples for short (30 s) and **(F)** Longer (120 s) film exposure time.

FIGURE 2: *Detecting CAI immunoreactivity in urine samples from newborns.* **(A)** Gestation (mean indicated by continuous line). **(B)** Age at urine collection (mean indicated by continuous line). **(C)** Blood hemoglobin (mean indicated by continuous line); normal range in neonates is

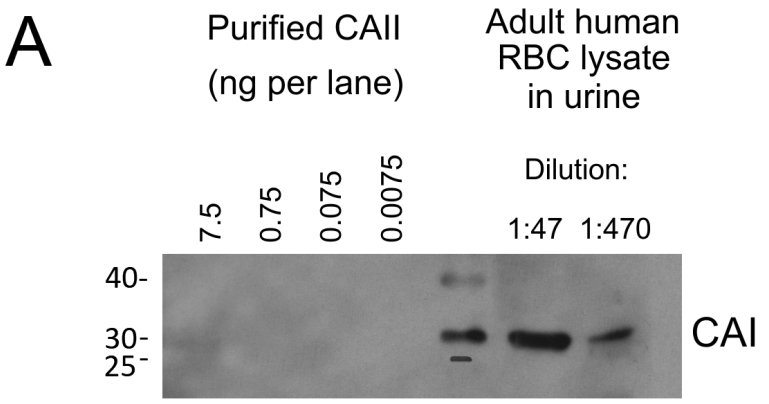
133-199 g/L. **(D)** Ratio of urine protein to urine creatinine. Dashed line indicates 95th percentile expected for this age-group. **(E)** Result of ELISA performed against CAI on 50 µL sample of undiluted urine. Dashed line shows background signal measured in wells with no urine (PBS-containing). Samples shaded grey produced detectable CAI immunoreactivity. Absorbance was converted to amount of CAI per well, based on a calibration curve shown in Figure S1. **(F)** Western blot performed on 30 µL sample of undiluted urine. Note, samples 9, 13 and 16 had insufficient urine for western blotting.

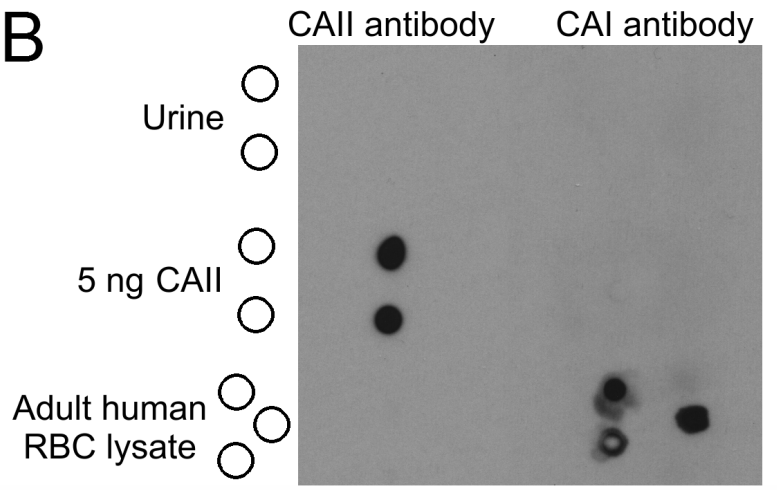
FIGURE 3: *Relating urinary CAI to clinical information.* **(A)** Stratification of neonates based on phototherapy status, C-reactive peptide (CRP) levels, and serum bilirubin (SBR). **(B)** Scatter plot of urinary CAI signal versus SBR. Absorbance was converted to amount of CAI per well, based on a calibration curve shown in Figure S1. **(C)** CAI signal (ELISA) categorized by neonate group. Statistical test: comparison to background signal (dashed line) by two-sided t-test of log-transformed data. **(D)** CAI signal (ELISA) versus age (in days) at which urine sample was obtained.

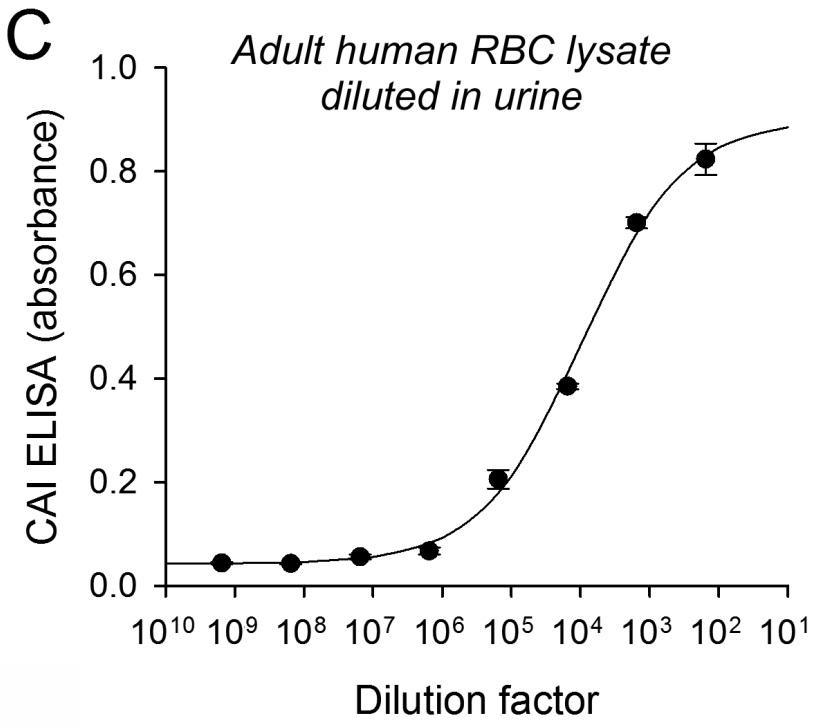
FIGURE 4: *Neonatal urine proteomics.* **(A)** List of 21 proteins that were present in urine samples and also in the top tertile of proteins in the RBC proteome. Among these, CAI had the highest mean level and sample-to-sample spread. Abundance was normalized to µg of total peptide. **(B)** Volcano plot identifying proteins with significantly different levels between CAI-immunopositive (N=16) and CAI-immunonegative (N=9) urine samples. Proteins highlighted in red are in the top tertile of proteins found in RBCs. Shading of non-RBC proteins is proportional to the proteins' abundance. The CAI-immunopositive group was enriched in 16 non-RBC specific proteins, including heme-binding protein 2, SPARC-like protein 1, zinc-α-

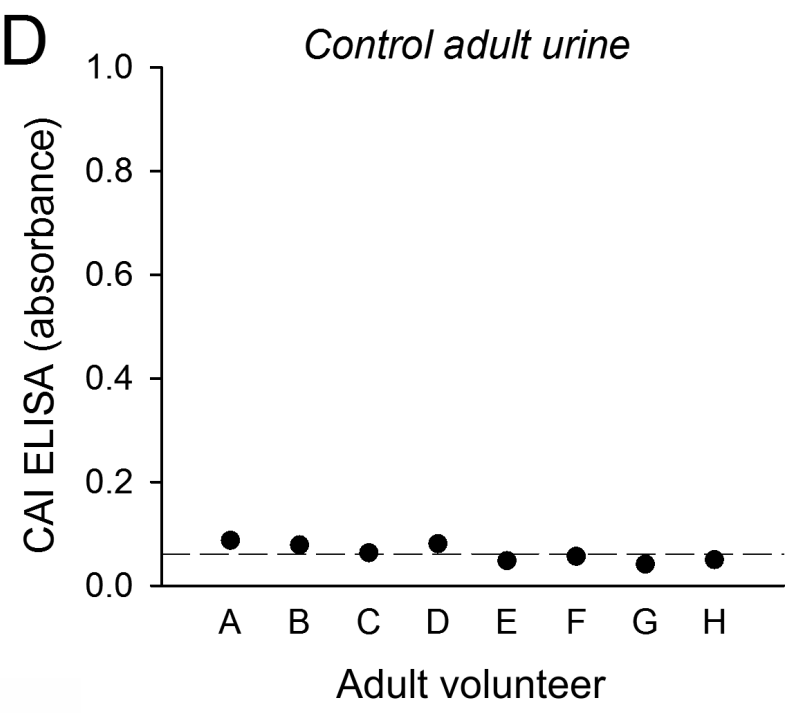
1-glycoprotein, cathepsin Z, calreticulin and L-selectin precursor. The CAI-immunonegative group was enriched in 23 non-RBC specific proteins, including calcyphosin, chondrolectin precursor, Lynx-1, platelet glycoprotein VI, tenascin X and IGF-like family receptor 1. **(C)** Label-free quantification (LFQ) of RBC-related proteins (normalized to urine volume), grouped by neonate category. Levels of CAI and PGK1 were significantly different (one-way ANOVA; *P<0.05) but CAI manifested larger absolute changes.

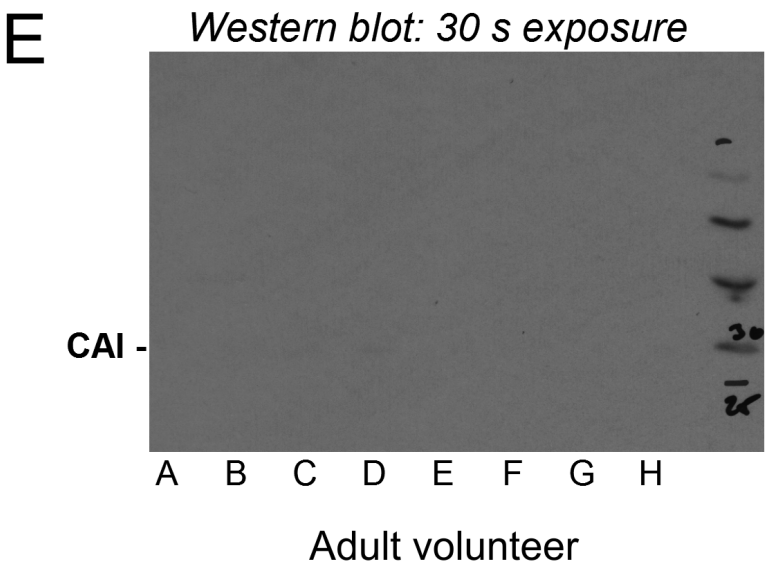
FIGURE 1


A
Purified CAII
(ng per lane)
Adult human
RBC lysate
in urine
Dilution:
7.5
0.75
0.075
0.0075
1:47
1:470
40-
30-
25-
CAI


B
CAII antibody
CAI antibody
Urine
5 ng CAII
Adult human
RBC lysate


C
Adult human RBC lysate
diluted in urine
CAI ELISA (absorbance)
1.0
0.8
0.6
0.4
0.2
0.0
10^10 10^9 10^8 10^7 10^6 10^5 10^4 10^3 10^2 10^1
Dilution factor


D
Control adult urine
CAI ELISA (absorbance)
1.0
0.8
0.6
0.4
0.2
0.0
A B C D E F G H
Adult volunteer


E
Western blot: 30 s exposure
CAI -
A B C D E F G H
Adult volunteer

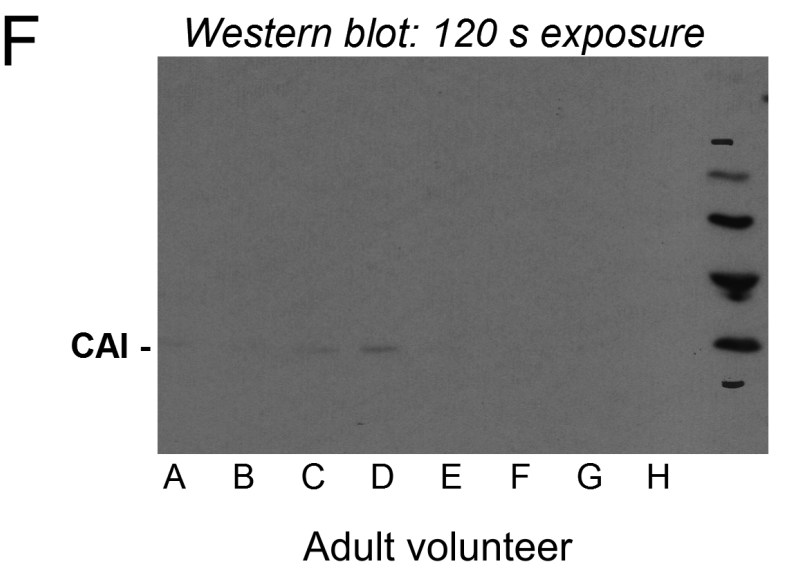

F
Western blot: 120 s exposure
CAI -
A B C D E F G H
Adult volunteer

FIGURE 2

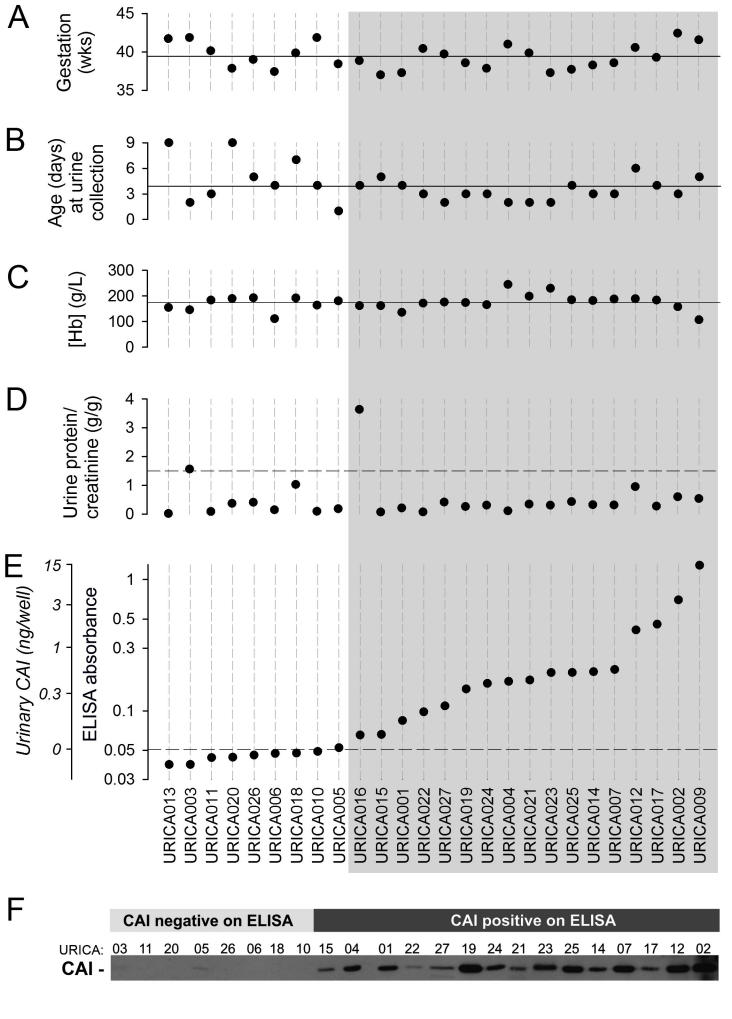

A
Gestation (wks)
45
40
35
B
Age (days) at urine collection
9
6
3
0
C
[Hb] (g/L)
300
200
100
0
D
Urine protein/ creatinine (g/g)
4
3
2
1
0
E
Urinary CAI (ng/well)
ELISA absorbance
15
3
1
0.3
0
1
0.5
0.3
0.1
0.05
0.03
URICA013
URICA003
URICA011
URICA020
URICA026
URICA006
URICA018
URICA010
URICA005
URICA016
URICA015
URICA001
URICA022
URICA027
URICA019
URICA024
URICA004
URICA021
URICA023
URICA025
URICA014
URICA007
URICA012
URICA017
URICA002
URICA009
F
CAI negative on ELISA
CAI positive on ELISA
URICA: 03 11 20 05 26 06 18 10 15 04 01 22 27 19 24 21 23 25 14 07 17 12 02
CAI -

FIGURE 3

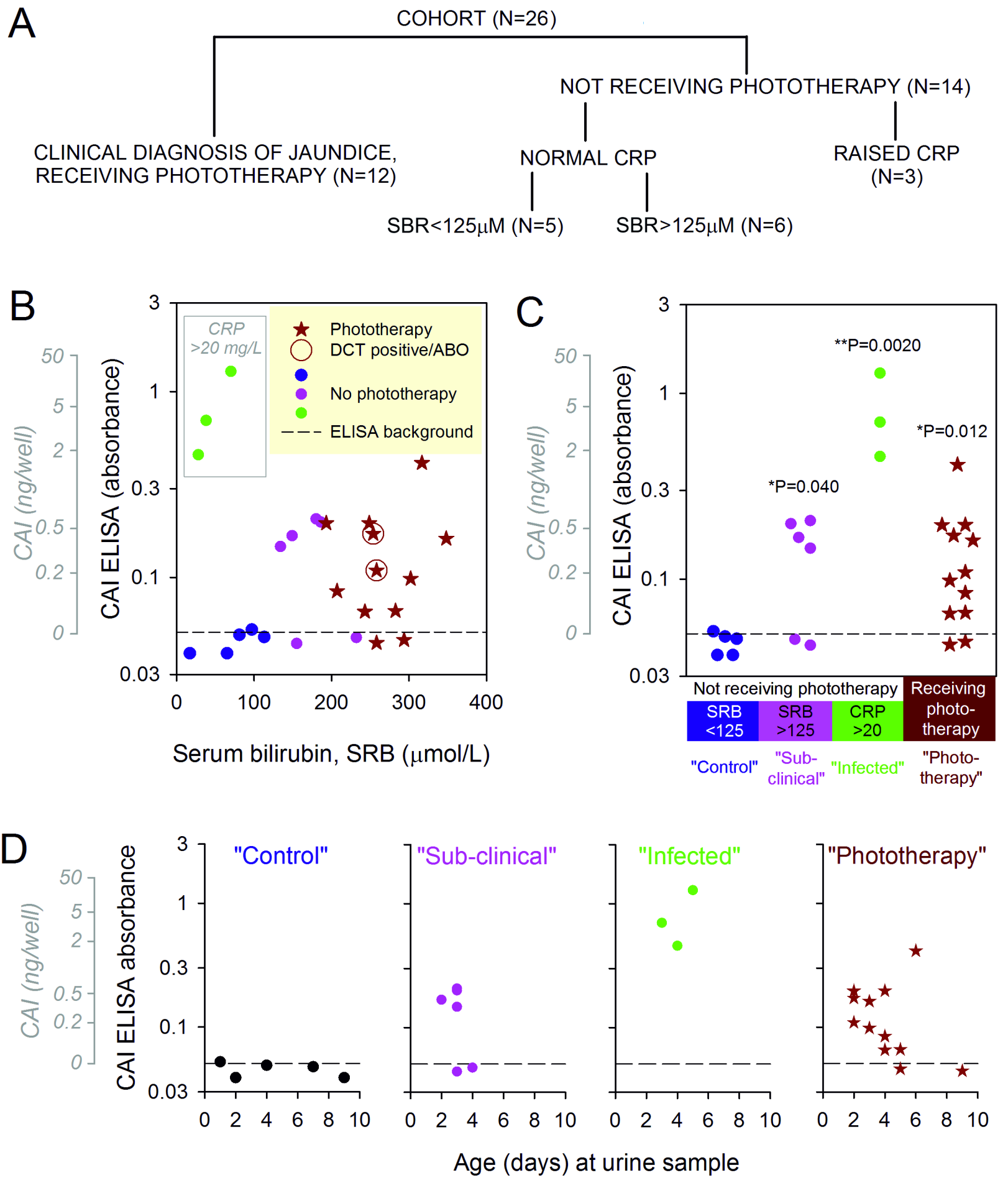
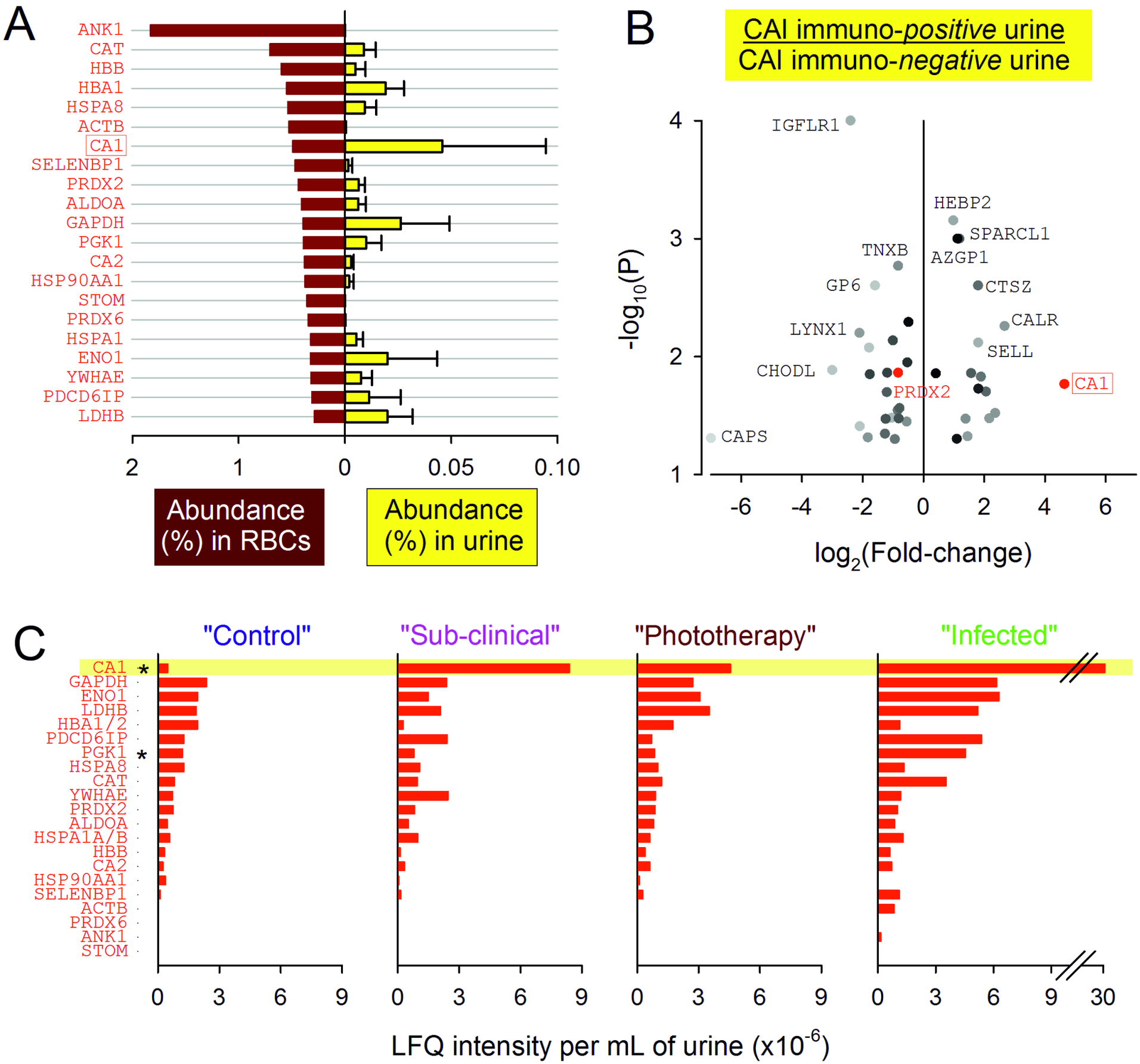

A
COHORT (N=26)
NOT RECEIVING PHOTOTHERAPY (N=14)
CLINICAL DIAGNOSIS OF JAUNDICE, RECEIVING PHOTOTHERAPY (N=12)
NORMAL CRP
RAISED CRP (N=3)
SBR<125μM (N=5)
SBR>125μM (N=6)
B
CRP >20 mg/L
Phototherapy
DCT positive/ABO
No phototherapy
ELISA background
CAI ELISA (absorbance)
CAI (ng/well)
Serum bilirubin, SRB (μmol/L)
C
**P=0.0020
*P=0.012
*P=0.040
Not receiving phototherapy
Receiving photo-therapy
SRB <125
SRB >125
CRP >20
"Control"
"Sub-clinical"
"Infected"
"Photo-therapy"
D
"Control"
"Sub-clinical"
"Infected"
"Phototherapy"
CAI ELISA absorbance
CAI (ng/well)
Age (days) at urine sample

FIGURE 4


A
ANK1
CAT
HBB
HBA1
HSPA8
ACTB
CA1
SELENBP1
PRDX2
ALDOA
GAPDH
PGK1
CA2
HSP90AA1
STOM
PRDX6
HSPA1
ENO1
YWHAE
PDCD6IP
LDHB
2
1
0
0.05
0.10
Abundance (%) in RBCs
Abundance (%) in urine
B
CAI immuno-positive urine
CAI immuno-negative urine
IGFLR1
HEBP2
SPARCL1
TNXB
AZGP1
GP6
CTSZ
CALR
LYNX1
SELL
CHODL
CA1
PRDX2
CAPS
-log10(P)
log2(Fold-change)
C
"Control"
"Sub-clinical"
"Phototherapy"
"Infected"
CA1 *
GAPDH
ENO1
LDHB
HBA1/2
PDCD6IP
PGK1 *
HSPA8
CAT
YWHAE
PRDX2
ALDOA
HSPA1A/B
HBB
CA2
HSP90AA1
SELENBP1
ACTB
PRDX6
ANK1
STOM
LFQ intensity per mL of urine (x10-6)

SUPPLEMENTAL METHODS

Power calculations. For a normal neonatal haematocrit of 56% (1-3 day neonates), 5% haemolysis would release ~100 mg of CAI per liter of plasma, assuming an RBC CAI concentration of 2 g/L *(1, 2)*. For a full-term neonate glomerular filtration rate of 39 ml/min/1.73 m^2 (~8 ml/min) *(3)* and a ultrafiltrate/plasma CA concentration ratio of 0.5 (for a molecule of Stokes radius 25 Å *(4-6)*), the urinary output of CAI is predicted to peak at ~0.4 mg/min *(2)*,. At urine flows of 50-300 µL/min, urine samples are expected to yield a detectable signal with sensitive immunoreactivity assays *(7)*. To resolve a 10-fold difference in CAI immunoreactivity, assuming a S.D. equal to the mean signal in the positive group and sampling ratio of 1, >20 babies were required.

Preparation of samples for testing primary antibody cross-reactivity. Stock solution of purified bovine CAII (Sigma-Aldrich) was diluted in adult human urine. Human RBC-lysate was prepared by diluting 10 µl of blood (hematocrit 45%) in 200 µl of adult urine (47-fold dilution), followed by freeze-thaw lysis.

Total protein measurement. Detergent Compatible Bradford Assay Reagent (Pierce) was used to determine total protein concentration in urine (three technical repeats each). Per well, 5 µl of urine was mixed with 150 µl of Bradford reagent, incubated for 10 min at room temperature, followed by absorbance measurements at 595 nm (Biotek ELx800 spectrophotometer).

Western blotting. 30µl urine was mixed with 10µl of 4x reducing Laemmli buffer (Bio-rad), and boiled for 5 minutes. The mixture was loaded onto polyacrylamide gel (30µl of urine/lane) for PAAG-electrophoresis and Western blotting using PVDF membrane. Membranes were then blocked in 5% skimmed milk prepared in 0.1% PBST for 1 hr at room temperature. Membranes were incubated overnight at 4°C with primary goat anti-human CAI polyclonal antibody (R&D Biosystems, Novus) diluted in 1:1000 in blocking buffer. Next day, the membranes were washed 4x in PBST for 30 min, and incubated with HRP-conjugated rabbit anti-goat IgG (H+L) secondary antibody (Thermofisher Scientific) diluted 1:10,000 in blocking buffer (1 hr at room temperature), and then washed. Membranes were incubated in ECL Western Blotting Substrate (Pierce) for 1 min and developed on X-ray films (GE Healthcare Amersham Hyperfilm).

Dot blot. Isolated bovine CAII (Sigma) was diluted in adult human urine (0.5 µg/200µl urine). Human RBC-lysate was prepared by diluting 10 µl of blood in 200 µl of urine, followed by lysis by freeze-thaw cycle. 2 µl aliquots of urine, bovine CAII-containing urine or RBC-lysate prepared in urine were pipetted onto nitrocellulose membrane (GE Healthcare Amersham), and air-dried. Membranes were blocked in 5% milk in PBST for 1 hr and incubated for 1 hr in room temperature in the presence of either primary goat anti-human CAI polyclonal antibody (R&D Biosystems, Novus) diluted in 1:1000 in blocking buffer, or rabbit anti-human CAII polyclonal antibody (Novus) diluted in 1:1000. Membranes were washed (4x in 30 minutes) and incubated for 1 hr with HRP-conjugated secondary antibodies diluted 1:10000 in blocking buffer (rabbit anti-goat IgG (H+L) secondary antibody, Invitrogen, or donkey anti-rabbit IgG (H+L) secondary antibody, Novus). After a second round of washing, membranes were developed using ECL Western Blotting Substrate (Pierce).

Urinary creatinine. Measurements of urinary creatinine were performed in a 96-well plate using a colorimetric assay kit (Cayman Chemical) according manufacturer's recommendation. Urine was diluted 10-fold in saline and 15 µl were aliquoted per well. Measurements were performed in duplicates. 150 µl of alkaline picrate solution was

added per well and incubated for 10 minutes on a shaker. This was followed by the initial measurement of absorbance at 490nm (Biotek ELx800 spectrophotometer). In the second step, 5µl of acid solution was added to samples, plate was incubated for another 20 min, and the final absorbance measured again at 490nm.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Urine aliquots were loaded onto Microcon 10kD centrifugal filters (Merck Millipore, MRCPRT01), concentrated by centrifugation and digested by trypsin using a Filter-Aided Sample Preparation (FASP) protocol. Dried gel digest peptide extracts were solubilised (20µl, 0.1% trifluoroacetic acid, TFA) and transferred to auto sampler vials for LC-MS/MS analysis. Tryptic peptides were separated using an Ultimate 3000 RSLC nano liquid chromatography system (Thermo Scientific) coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) via a Proxeon nano-spray source. Sample volumes corresponding to 1.0 µg of protein digest, as determined by a fluorimetric peptide concentration assay (Pierce), were loaded onto a trap column (Acclaim PepMap 100 C18, 100µm × 2cm) at a flow rate of 8 µL/min in 2% acetonitrile, 0.1% TFA. Peptides were then eluted on-line to an analytical column (Acclaim Pepmap RSLC C18, 75µm × 50cm) and separated using a stepped 120 minute gradient of 4-65% buffer B (buffer A: 2% acetonitrile, 0.1% formic acid buffer, buffer B: 80% acetonitrile, 0.1% formic acid). Eluted peptides were analysed by the LTQ XL operating in positive polarity using a data-dependent acquisition mode. Ions for fragmentation were determined from an initial MS1 survey scan at 30,000 resolution (at m/z 200), followed by Ion Trap CID (collisional induced dissociation) of the top 6 most abundant ions. MS1 and MS2 scan AGC targets set to 1e6 and 1e4 for a maximum injection time of 500 ms and 100 ms respectively. A survey scan m/z range of 350-1800 was used, with a normalised collision energy set to 35%, charge state rejection enabled for +1 ions and a minimum threshold for triggering fragmentation of 500 counts.

LC-MS/MS analysis. Data was processed using the MaxQuant software platform (v1.5.8.3), with database searches carried out by the in-built Andromeda search engine against a UniProt human database (version 20170202). A reverse decoy database approach was used at a 1% FDR for peptide spectrum matches and protein identifications. Search parameters included: maximum missed cleavages set to 2, fixed modification of cysteine carbamidomethylation and variable modifications of methionine oxidation and protein N-terminal acetylation. Label-free quantification was enabled with an LFQ minimum ratio count of 2. 'Match between runs' function was used with match and alignment time windows of 1 and 20 minutes respectively.

References

1. Backman U, Danielsson B, Wistrand PJ. The excretion of carbonic anhydrase isozymes CA I and CA II in the urine of apparently healthy subjects and in patients with kidney disease. Scand J Clin Lab Invest 1990;50:627-33.
2. Robinson JR. Urinary excretion of carbonic anhydrase: A simple test for the detection of intravascular haemolysis. Journal of clinical pathology 1950;3:142-5.
3. Heilbron DC, Holliday MA, al-Dahwi A, Kogan BA. Expressing glomerular filtration rate in children. Pediatric nephrology 1991;5:5-11.
4. Maren TH. Carbonic anhydrase: Chemistry, physiology, and inhibition. Physiol Rev 1967;47:595-781.
5. Sly WS, Hu PY. Human carbonic anhydrases and carbonic anhydrase deficiencies. Annu Rev Biochem 1995;64:375-401.
6. Supuran CT. Carbonic anhydrases: Novel therapeutic applications for inhibitors and activators. Nature reviews Drug discovery 2008;7:168-81.
7. Wahlstrand T, Knuuttila KG, Wistrand PJ. A radioimmunosorbent technique for the assay of b- and c-types of carbonic anhydrase in human tissues. Scand J Clin Lab Invest 1979;39:503-9.
8. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods 2009;6:359-62.
9. Cox J, Hein MY, Luber CA, Paron I, Nagaraj N, Mann M. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed maxlfq. Mol Cell Proteomics 2014;13:2513-26.

SUPPLEMENTAL FIGURES

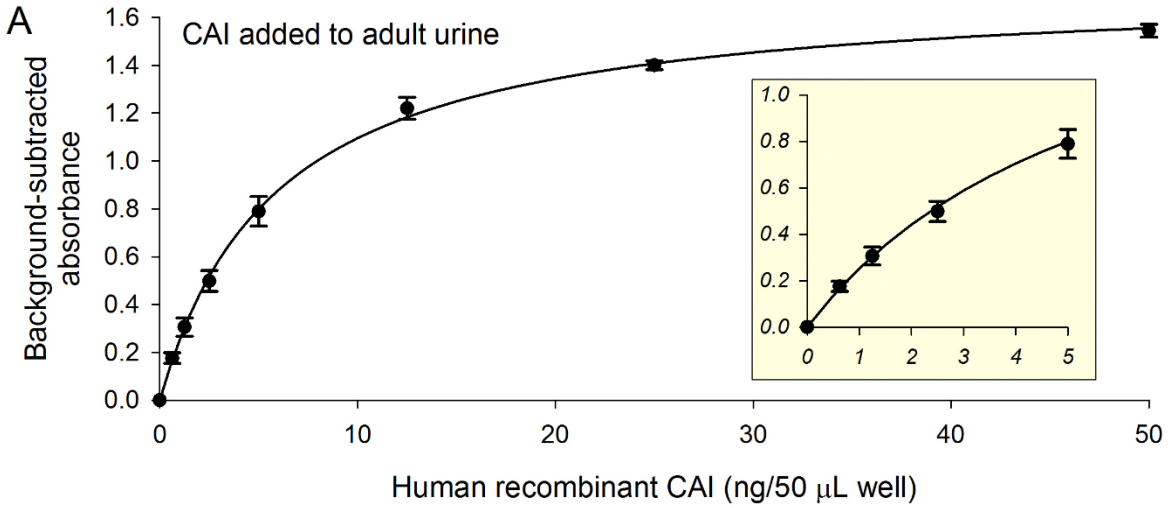


Figure S1: ***Testing linearity of CAI ELISA with recombinant CAI.*** Calibration curve for human recombinant CAI (hrCAI; R&D Biosystems) produced by serial dilution of 100 ng/µl hrCAI in human adult urine and repeated for a further 3 urine samples. Final CAI concentrations were: 50, 25, 12.5, 5, 2.5, 1.25, 0.625 and 0 ng hrCAI per 50 µl well. ELISA assay was performed as described by the Methods. Absorbance, subtracted for background (urine sample with no added hrCAI), is plotted on a linear axis. Note the linearity of absorbance over the range up to ~5 ng hrCAI / 50 µl well. Inset replots curve for range 0-5 ng hrCAI per 50 µl well.

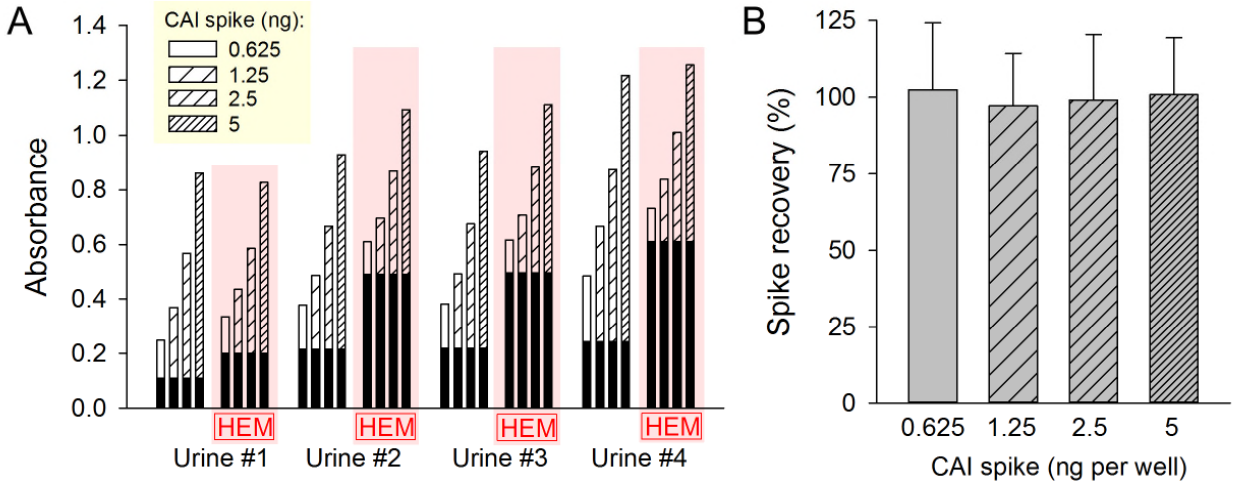


Figure S2: ***Spiking/recovery test of the CAI assay.*** **(A)** A spiking/recovery study was performed on urine obtained from four adults. Each urine sample was split into two groups, and one (indicated as "HEM" for hemolysate) was enriched with filtered hemolysate to raise background CAI signal by an average of 0.3 absorbance units. Hemolysate was produced from venous blood collected from a healthy adult volunteer. 1 ml of blood was spun-down and the supernatant was discarded. The blood cell pellet was washed three times with ice-cold calcium-free PBS and freeze-thaw lysed in PBS. Lysate was filtered through Amicon Ultra centrifugal filters of cut-off 100kDa to remove most substances that would not normally pass the glomerular barrier *in vivo*. Filtered hemolysate was 75x-diluted in PBS for stock, and subsequently diluted in urine (150x) to a final dilution of 11,250-fold. Samples were spiked with human recombinant CAI (hrCAI), to 0.625, 1.25, 2.5 or 5.0 ng per well (50 µL), followed by the ELISA protocol, as described in the Methods. **(B)** Spike recovery was calculated as the measured increase in CAI (in units of ng per well) upon addition of the 'spike', relative to the actual amount of hrCAI added (ng/well), and expressed as a percentage. For this calculation, absorbance was converted to CAI (in units of ng/well) using a calibration curve performed on the respective urine samples for a range of hrCAI (0-50 ng/well). Recovery was, on average, 98%. Data shown as mean±S.D.

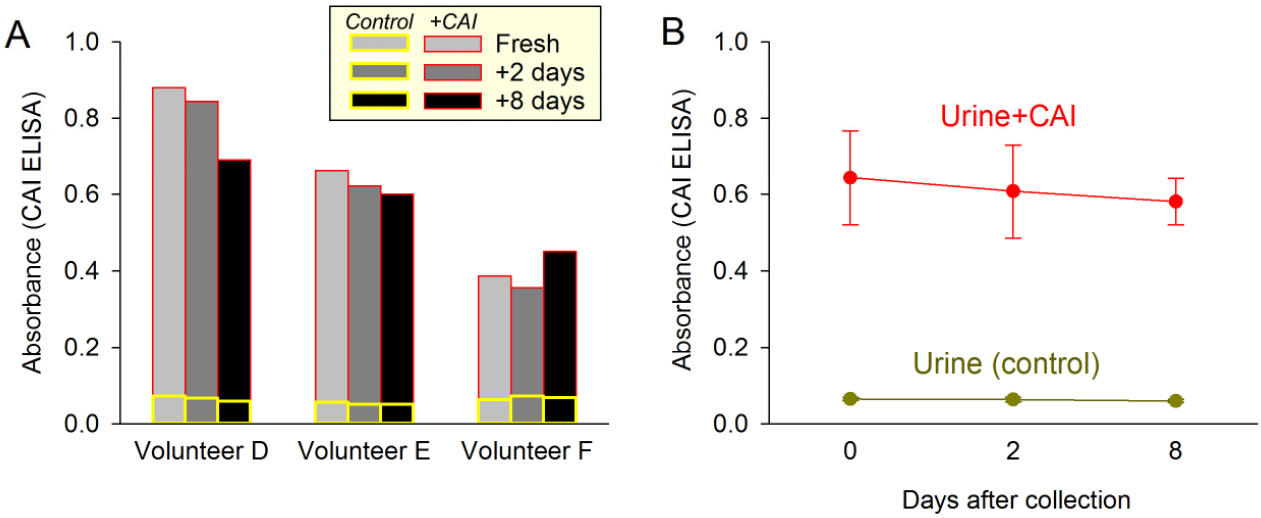


Figure S3: Testing *the precision of the CAI assay.* Urine was obtained from three non-hemolysing adult volunteers (female, male, male), and split into a control (blank urine) and CAI-spiked group. To produce CAI-spiked urine, human recombinant CAI (hrCAI) was added to levels that are expected to yield ELISA absorbance in the range 0.4-0.8 (on average, 3 nM CAI added per well). All samples were then aliquoted for three tranches of experiments performed at different timepoints. The first tranche was used to determine ELISA absorbance on the day of urine collection. The second and third tranches were used for ELISA measurements after 2 and 8 days of cold storage. **(A)** Absorbance measurements for each urine sample for repeated measures over 8 days. **(B)** Mean±SEM of ELISA absorbance in CAI-spiked and control urine.

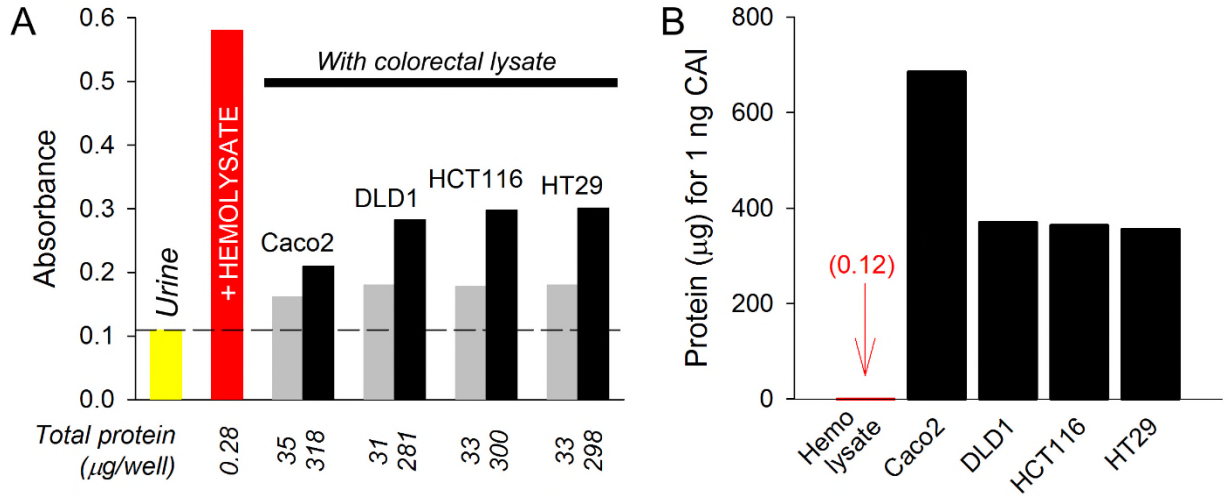


Figure S4: *Comparing the yield of CAI in hemolysate versus lysates prepared from colorectal cell lines.* **(A)** CAI measurements were performed by ELISA on adult urine *(yellow)*, urine enriched with hemolysate at 750x dilution (see Fig S2) *(red)*, and urine enriched with lysates of four colorectal cell lines (Caco2, DLD1, HCT116, HT29) at two dilutions *(gray and black)*. Colorectal lysates were prepared from cells grown in standard DMEM with 10% fetal calf serum in a 5% CO_2/air atmosphere incubator (37°C). Briefly, upon reaching confluency, cells were lysed in RIPA lysis buffer (Cell Signalling Technologies), stored at −80°C, and diluted in urine when required. The total protein content of stock hemolysate (in PBS) and colorectal cell lysates (in RIPA) was measured using the BCA protein assay (Pierce, Thermofisher). Absorbance was measured at 562nm by the Cytation 5 plate reader (Bio-Tek). Protein concentration (µg/well) was calculated for each sample, and shown below the x-axis. **(B)** Absorbance was first converted to CAI (ng/well) using a calibration curve (see Fig S2), and then replotted as the ratio of total protein (µg) per ng of CAI. This provides information about the amount of protein (hemolysate or colorectal cell lysate) required to yield 1 ng of CAI, i.e. a level that produces a detectable signal by the assay. ~5000-times more colorectal cell lysate than hemolysate is required to produce this level of CAI.

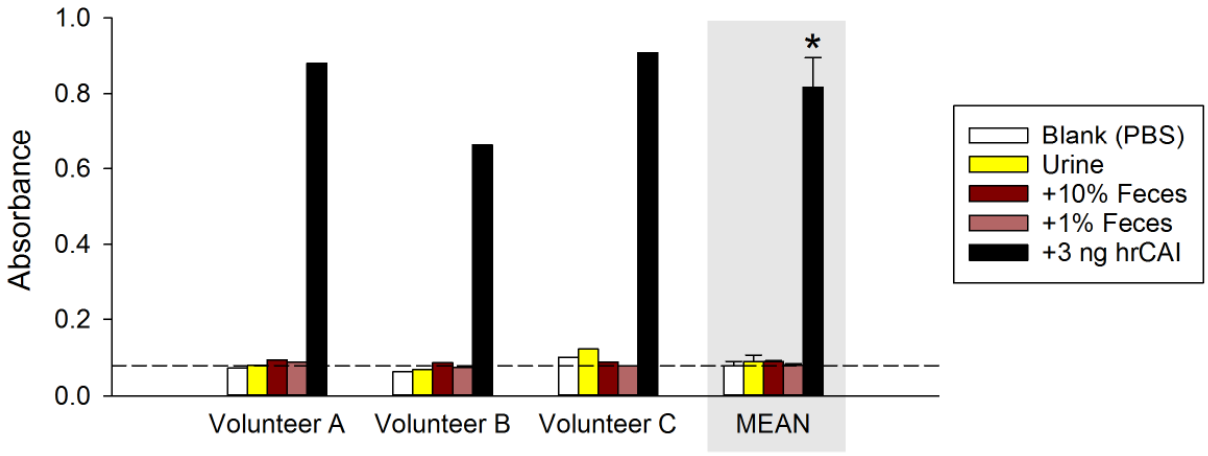


Figure S5: ***Fecal contamination does not produce a false-positive signal of CAI.*** Urine and a sample of feces were collected from three adult volunteers (female, male, female). To produce feces-contaminated urine, a 1 ml sample of fecal matter was added to 9 ml urine, followed by vigorous mixing and two rounds of ultracentrifugation (9000 rpm, 10 min, 4°C). The clear supernatant was used for ELISA measurements. These measurements were also performed on a 10-fold dilution of contaminated urine i.e. 1% feces, and on urine that had no added fecal matter. As a positive control for CAI, 3 ng of hrCAI was added to urine. Only the positive control produced a significant ELISA signal, indicating that fecal contamination as large as 10% (v/v) cannot yield a CAI signal to explain positive results in hemolysing neonates.

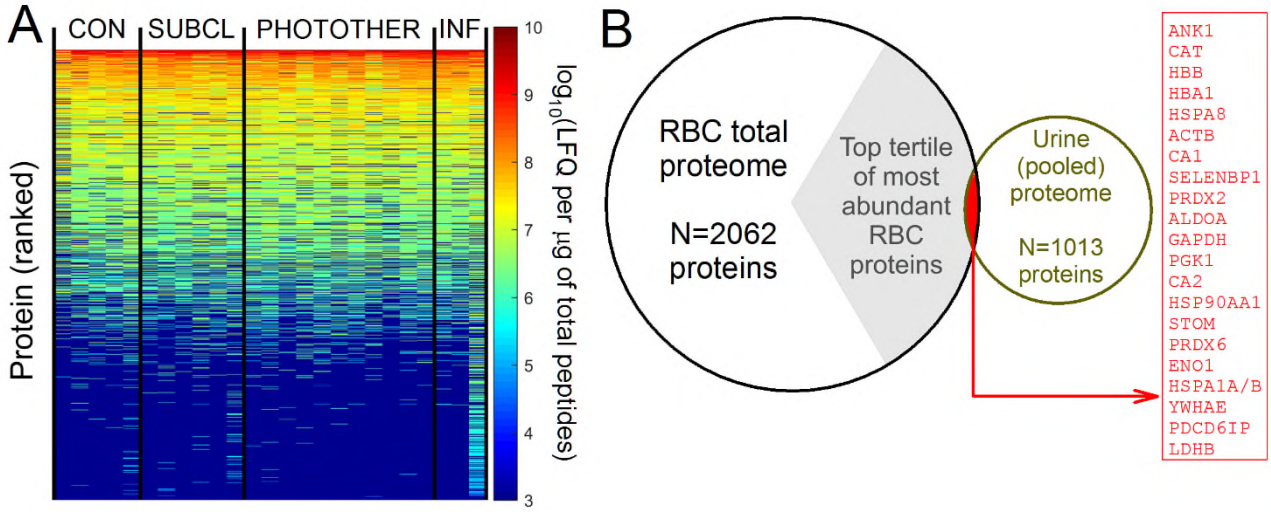


FIGURE S6: ***Neonatal urine proteomics.*** **(A)** Proteomics of neonatal urine, ranked by copy number and stratified by group. A full list of proteins identified is given in Table S1. **(B)** A comparison of the urinary proteome against a list of the most abundant peptides in the RBC proteome (top tertile) identifies 21 protein-hits.