SUPPLEMENTARY METHODS

Endogenous Ouabain (EO) measurement. Plasma samples are pre-extracted with methanol, dried and reconstituted with 0.1% trifluoroacetic acid for extraction using preconditioned C18 Bond Elut columns (Varian, Inc., Palo Alto, California, USA). Following several water washes and one wash with 2.5% acetonitrile, EO is eluted by 25% acetonitrile. These steps are critical for two main reasons: first, the recovery of endogenous ouabain from the column can be variable (40–90%, Ferrandi, unpublished observation) if the preconditioning is not correct, and second, the 2.5% acetonitrile wash reduces the highly polar interferants present in some extracts. Rabbit polyclonal antiouabain antisera of high titer prepared by Prassis Sigma Tau, Scientific Institute in the late 2000s and provided by one of us were used in the Milan radioimmunoassay. These antiouabain antisera possessed panels of cross-reactivity very similar to digitalis or steroidal molecules, and both antisera recognized (in addition to ouabain) two aglycones structurally related to ouabain, that is, ouabagenin and strophanthidin-K. The cross-reactivity of the two antisera with the purified human plasma EO differed slightly from each other but was similar within experimental error to ouabain (Hamlyn, unpublished observation). The possibility that the antisera used in our radioimmunoassay can recognize the human placental inhibitor of Na+–K+–ATPase, whose suggested structure resembles that of a bufadienolide (1,2) is very unlikely because the two ouabain antisera we used show very weak cross-reactivity with the bufadienolides (<1%). Moreover, the sample extraction process we employ precludes the presence of bufadienolides in the endogenous ouabain assay because they are not sufficiently polar to be eluted by 25%

acetonitrile under our conditions. Another variable is the method used to separate bound from free-labeled ouabain. We employ a filtration technique using glass fiber filters and a cell harvester that allows for rapid separation of free vs. antibody-bound label. The rapidity of the separation is important in the radioimmunoassay format because bound 3H-ouabain dissociates fairly rapidly from the antisera we use. Hence, it is crucial that the separation method is not only rapid but also highly reproducible across a large number of samples. For the Milan EO radioimmunoassay system, the intra assay and interassay coefficient of variation were approximately 5 and 9%, respectively, and remain remarkably steady over the years. When all aspects of the radioimmunoassay method are respected, all tissues from rats and humans including plasma contain measurable and reproducible amounts of endogenous ouabain by radioimmunoassay and ATPase assay, both before and after HPLC fractionation (3).

Critically Ill Patients (intensive care unit (ICU) cohort): This was a prospective observational study conducted at a single center from July 2004 to July 2005. The protocol was approved by the institutional review board of the University of Maryland. Adult patients admitted to the ICU were eligible if the patient did not have an acute cardiac condition (acute coronary syndrome, congestive heart failure, or arrhythmia). Disease severity was scored according to Physiology and Chronic Health Evaluation (APACHE II) system42 with higher values indicating more severe illness. Data related to NT-proBNP has previously been published.

Cardioplegia (4) is a pharmacologically induced electromechanical heart arrest used to achieve the ideal conditions for cardiac surgery. A number of constituents

have been added in various concentrations in different cardioplegic solutions. Potassium and magnesium induce pharmacological arrest. Steroids, glucose and high energy phosphates are used as membrane stabilisers. Glucose, mannitol and sorbitol are used as hyperosmotic solutions, while bicarbonate, phosphate and THAM (tris-hydroxymethyl aminomethane) can be added as buffering agents.

Custodial Cardioplegia. Custodiol HTK (Histidine - Tryptophan - Ketoglutarate) Solution is used by Leading Cardiac Surgery Centers as a cardioplegic solution worldwide. A product approved by US FDA as a Medical Device and being a Multi-Organ solution is also the solution of choice as an organ preservation solution for Heart Transplantation.

The advantages of Custodiol cardiplegia are:

- Low potassium concentration: minimize risks;
- Low sodium concentration: rapid and homogeneous cooling due to low viscosity;
- Extended buffer capacity: good recovery of myocardial function;
- Tryptophane for membrane integrity: excellent ischemic tolerance;

Composition: 1000ml of Custodiol HTK solution contains		
0.8766g	Sodium Chloride	15.0 mmol
0.6710g	Potassium Chloride	90 mmol
0.8132g	Magnesium Chloride	
6.000	H2O	4.0 mmol
3.7733g	Histidine Hydrochloride Monohydrate	18.0 mmol
27.9289	Histidine	180.0 mmol
0.4045g	Tryptophan	2.0 mmol
5.4651g	Mannitol	30.0 mmol
0.0022g	Calcium Chloride	
2.000	H2O	0.015 mmol
0.1842g	Potassium Hydrogen 2 Ketoglutarate	1.0 mmol

Buckberg's Solution. Buckberg is one of the most used solutions for blood cardioplegia (5). A blood vehicle for cardioplegic solutions delivery blends onconicity, buffering, rheology, and antioxidant benefits with its capacity to augment oxygen delivery and ability to 'resuscitate' the heart, prevent ischemic injury, and limit reperfusion damage. The cardioprotective potential of Buckberg's solution is represented by the synergistic effect of its different components:

- hyperkalemia: induction and maintenance of cardioplegic arrest
- hypocalcemia: avoidance of mitochondrial calcium overload and prevention of irreversible myocyte injury.
- tris buffer: prevention of tissue acidosis
- hyperosmolarity and hyperglycemia: prevention of myocardial edema
- glutamate and aspartate: these amino acids replenish key Krebs-cycle depleted during ischemia by enhancing aerobic metabolism and reparative processes.

Buckberg's solution composition.

KCl	15 meq/l
pH	7.5-7.6
Creatin-bi-phosphate	0.15-0.25 nmol/l
Citrates	25.6 nmol/l
Glucose	5% 4 g/l
THAM (tris-hydroxymethyl aminomethane)	200 ml

Experimental model. Care and husbandry of rats complied with the European Directives nº 86/609 and with the Italian Law (DL116, January 27, 1992). The authorization for animal use in Prassis Sigma-Tau laboratories was obtained from the Italian Health Authority. Ouabain infused Rats (OHR) were generated by subcutaneous ouabain infusion (Sprague-Dawley rats), of male rats (5-6 week old, weighing 120-130 g) with osmotic mini-pumps (Alzet, Charles River, Calco, Italy) containing a ouabain-saline solution that slow-released 15 μg/kg/day ouabain (Sigma-Aldrich, n=7) with osmotic mini-pumps for 8 weeks as previously described (6). Normotensive control rats (n=7) received sterile saline solution through osmotic mini-pumps. Systolic blood pressure (SBP) and heart rate (HR) were recorded weekly at the tail by plethysmography (BP recorder, U. Basile, Varese, Italy). The initial SBP of controls and OHR rats was comparable (average 130-135 mmHg). After 8 weeks of ouabain infusion, double plasma EO, SBP rose significantly in OHR rats (171±1.3 mmHg) over controls (150±1.9 mmHg). HR was not affected in either group (controls 364±4.3, OHR 374±4.8 beats/min).

Biochemical assays for urinary parameters. Rats were housed in single metabolic cages and 24h-urine samples were collected and analyzed for total protein and creatinine excretion (kits from Sentinel Diagnostics, Milan, Italy). At sacrifice, rat plasma was collected for creatinine determination. Creatinine was quantified by the colorimetric Jaffè method which measures the colour formation with picric acid.

Immunohistochemistry. Immunofluorescence was performed on unfixed rat renal tissue embedded in OCT compound, snap-frozen and stored at -80°C.

Rat kidney sections were incubated with anti-nephrin (Progen) and anti-synaptopodin (Abcam) antibodies followed by appropriate secondary antibodies and developed.

Renal microsome preparations. Renal microsomes were prepared from OHR rats and normotensive controls. Kidneys were homogenized in 250 mM sucrose, 30 mM histidine, 5 mM EDTA pH 7.2, centrifuged at 6000g for 15 minutes. The supernatant was centrifuged at 48000g for 30 min. The pellet, containing renal microsomes, was resuspended in sucrose-histidine buffer and analyzed by Western blotting.

Podocyte isolation. Kidneys were taken from 7-10 day-old rats and glomerular podocytes were isolated by sieving then seeded in cultured flasks precoated with collagen type IV (Sigma) at 37°C in 5% CO2 atmosphere. On day 4-5, podocyte growth started and by day 8 glomeruli were detached using trypsin-EDTA and filtered through a 36 µm-mesh to eliminate glomeruli. Cell characterization was performed using markers of podocytes (nephrin, podocin, synaptopodin), epithelial (cytokeratin), smooth muscle (a-SMC) and endothelial cells (CD31). Second passage podocytes were seeded on flasks and incubated for 4 days with ouabain (10^{-11} – 10^{-8} M) and analyzed by Western blotting.

Western blotting. Samples were separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, blotted and incubated with specific antibodies against podocyte markers, such as nephrin (Progen), podocin (Sigma-Aldrich), synaptopodin (Progen Biotechnik), ZO-1 (Invitrogen) followed by 1h incubation with fluorescent secondary antibodies then analyzed by Odyssey

Infrared Imaging Detection System (LI-COR Biosciences). Actin (Sigma-Aldrich) was used for normalization (7-8).

SUPPLEMENTARY RESULTS

Critically Ill Patients-ICU cohort: the most common admission diagnoses to the ICU of the 54 (56% African American) patients studied were sepsis (43%), pneumonia (44%), and acute respiratory distress syndrome (37%, Table 4). AKI (37%) and hepatic dysfunction (17%) were common (Supplementary Table 4). Median EO concentration was 270 (IQR: 204 – 411) pmol/L. APACHE II score (11.8 ± 7.4, 14.9 ± 6.2, 19.9 ± 10.2, p=0.02, Supplementary Figure 1 A) and in-hospital mortality (IHM) (11%, 39, 44%, p=0.07, Supplementary Figure 1 C) increased with each EO tertile. Acute renal failure (11%, 39%, 61%, p=0.008) and acute hepatic dysfunction (6%, 0%, 44%, p=0.001) increased with each incremental tertile (Supplementary Table 5). EO concentrations were associated with increased serum creatinine (β=0.497, P < 0.001, Supplementary Figure 1 B), total bilirubin concentrations (β=0.438, P=0.001) and NT-proBNP (β=0.5, P < 0.001, Supplementary Figure 1 D). The associations remained independently significant after adjustments for covariates.

REFERENCES

1. Lopatin DA, Ailamazian EK, Dmitrieva RI, et al: Circulating bufodienolide and cardenolide sodium pump inhibitors in preeclampsia. *J Hypertens* 1999; 17:1179-1187

2 Lichtstein D, Steinitz M, Gati I, et al: Bufodienolides as endogenous Na+, K+-atpase inhibitors: Biosynthesis in bovine and rat adrenals. *Clin Exp Hypertens* 1998;20:573-579

3 Ferrandi M, Manunta P, Balzan S, et al: Ouabain-like factor quantification in mammalian tissues and plasma: Comparison of two independent assays. *Hypertension* 1997; 30:886-896

4 Zhidkov IL, Belianko IE, Sitnichenko NV, et al: Effect of perfusion volume of the Custodiol solution on the efficiency of cardioplegia in experiment. Anesteziol Reanimatol 2008; Sept-Oct (5):42-47

5 Dyson CW, Emerson RC, Buckberg GD. A hemodilution cardioplegia and a proposed delivery system. 1980. J Extra Corpor Technol 2008; 40:142-144

6 Ferrari P, Torielli L, Ferrandi M, et al: Pst2238: A new antihypertensive compound that antagonizes the long-term pressor effect of ouabain. *J Pharmacol Exp Ther* 1998; 285:83-94

7 Ferrandi M, Cusi D, Molinari I, et al: Alpha- and beta- adducin polymorphisms affect podocyte proteins and proteinuria in rodents and decline of renal function in human Iga nephropathy. *J Mol Med* 2010; 88:203-217

8 Ferrandi M, Molinari I, Barassi P, et al: Organ hypertrophic signaling within caveolae membrane subdomains triggered by ouabain and antagonized by PST 2238. *J Biol Chem* 2004; 279: 33306-33314

SUPPLEMENTARY TABLES AND FIGURE

Supplementary Table 1. Cardiac Surgery: clinical characteristics of the study participants

Combined surgery is valve repair/replacement joined with coronary-artery bypass or multiple valve replacement/repairs. Closure of inter-atrial septal defects, excision of intra-myocardial masses, aneurysmectomy and atrial fibrillation radiofrequency ablation are included in the category "other". Data are presented as mean ± SD. P EO and P creatinine are median (Inter Quartile Range).

	Pre Operative
SEX (f/m)	158/249
AGE (yrs)	61.7 ±13.8
BMI (kg/m^2)	25.0±4.01
EF (%)	57.0 ±10.5
Plasma Creatinine (mg/dL)	0.85 (0.76-0.98)
eGFR (ml/m/1.73)	75.8±20.16
EUROSCORE	4.7±4.92
ACEF	1,1±0.37
Plasma EO (pmol/L)	155 (107-233)
Surgical planning	
Mitral valve repair	129 (31.7%)
Other Valve Repair	82 (20.1%)
CABG:	52 (12.8%)
Combined surgery	82 (21.9%)
AAR	42 (10.3%)
Others	13 (3.2%)
Reintervenion	50 (12.3)
Prior Medical History	
Hypertension	183 (45.0%)
Chronic kidney disease	49 (12.0%)
Diabetes	42 (10.3%)
NYHA I,II,III,V (%)	24 51 23 2

Intra Operative	
Surgery time (min)	263.9±79.0
CPB (%)	379 (94.3%)
Custodial cardioplegia	323 (76,1%)
CPB time (min)	93.5±35.74
ACCT (min)	70.7±25.91
MAP (mmHg)	71.8±6.34
Post Operative	
Inotropes (n°)	169 (42.1%)
Diuretics (n°)	22 (5.5%)
IABP (n°)	15 (3.8%)
Transfusion (n°)	56 (22.5%)
Plasma EO (pmol/L)	254 (206.5-394)
Plasma Creatinine (mg/dl)	1.00 (0.82-1.38)
Troponin T peak	2.2±3.32
ICU stay (days)	2.9±4.79
LHS (days)	11.9±11.8
Outcames	
Severe AKI (%)	42 (10.3%)
RRT (%)	14 (3.4%)
IHM (%)	10 (2.35%)

Supplementary Table 2. Validation cohort clinical characteristics

Combined surgery is valve repair/replacement joined with coronary-artery bypass or multiple valve replacement/repairs. Closure of inter-atrial septal defects, excision of intra-myocardial masses, aneurysmectomy and atrial fibrillation radiofrequency ablation are included in the category "other". Data are presented as mean ± SD. P EO and P creatinine are median (Inter Quartile Range).

	Pre Operatve
SEX (f/m)	64/155
AGE (yrs)	61.9±11.78
BMI (kg/m^2)	24.9±4.14
EF (%)	56.0±9.48
Plasma Creatinine (mg/dL)	0.9 (0.76-0.98)
eGFR (ml/m/1.73)	86.2±17.63
EUROSCORE	3.4±3.31
ACEF	1.2±0.38
Plasma EO (pmol/L)	157.9 (115-210)
Surgical planning	
Mitral valve repair	28 (12.8%)
Other valve repair	84 (38.4)
CABG:	27 (12.3%)
Combined surgery	15 (6.8%)
AAR	65 (29.7%)
Others	0 (0%)
Previous cardiac surgery	11 (5.0%)
Prior Medical History	
Hypertension	144 (65.8%)
Chronic kidney disease	13 (5.9%)
Diabetes	36 (16.4%)
NYHA I,II,III,V (%)	18 60 22 0

Intra Operative	
Surgery time (min)	262.1±69.0
CPB (%)	210 (95.9%)
Custodial cardioplegia	199 (86,9%)
CPB time (min)	92.9±38.27
ACCT (min)	74.9±31.71
MAP (mmHg)	72.8±6.40
Post Operative	
Inotropes (n°)	87 (39.7%)
Diuretics (n°)	74 (33.8%)
IABP (n°)	15 (6.9%)
Transfusion (n°)	26 (11.9%)
Plasma EO (pmol/L)	240.9 (157)
Plasma Creatinine (mg/dl)	1.03 (196-317)
Troponin T peak	1.2±1.13
ICU stay (days)	2.2±4.20
LHS (days)	7.3±5.6
Outcames	
Severe AKI (%)	25 (11.4%)
RRT (%)	2 (0.9%)
IHM (%)	1 (0.5%)

Supplementary Table 3. Cumulative analysis. Multivariable Logistic Regression Analysis for severe acute kidney injury (AKI) the 9 identified Predictors

	B	S.E.	Wald	df	Sig.	Exp(B)	95%C.I. for exp(B)	
							Lower	Upper
sex(1)	-0,510	0,324	2,479	1	0,115	0,601	0,319	1,133
age	0,049	0,017	7,915	1	0,005	1,050	1,015	1,086
Redo-intervention(1)	0,837	0,405	4,262	1	0,039	2,310	1,043	5,114
hypertension(1)	0,237	0,340	0,486	1	0,486	1,268	0,651	2,470
diabetes(1)	0,504	0,369	1,867	1	0,172	1,655	0,803	3,408
EF (%)	-0,035	0,014	6,121	1	0,013	0,966	0,940	,993
eGFR basal	-0,019	0,009	4,215	1	0,040	0,981	0,964	,999
Combined intervention(1)	0,629	0,333	3,557	1	0,059	1,875	0,976	3,603
lgEO basal	2,820	0,623	20,489	1	0,000	16,773	4,947	56,869
Constant	-8,665	2,398	13,054	1	0,000	0,000		

(1) binary recoded

Supplementary Table 4. Patients with preoperative estimated glomerular filtration rate (eGFR) lower than 60 ml were excluded, 36 developed postoperative acute kidney infjury (AKI), 464 were negative

Area Under the Curve

	Area	SEM	p		CI 95%
Test Result Variable(s)					
EOSCORE	0.84	0.032	<0.0001	0.776	0.900
Clinical Model	0.75	0.040	<0.0001	0.676	0.832

Supplementary Table 5. Intensive care unit (ICU) cohort clinical characteristics

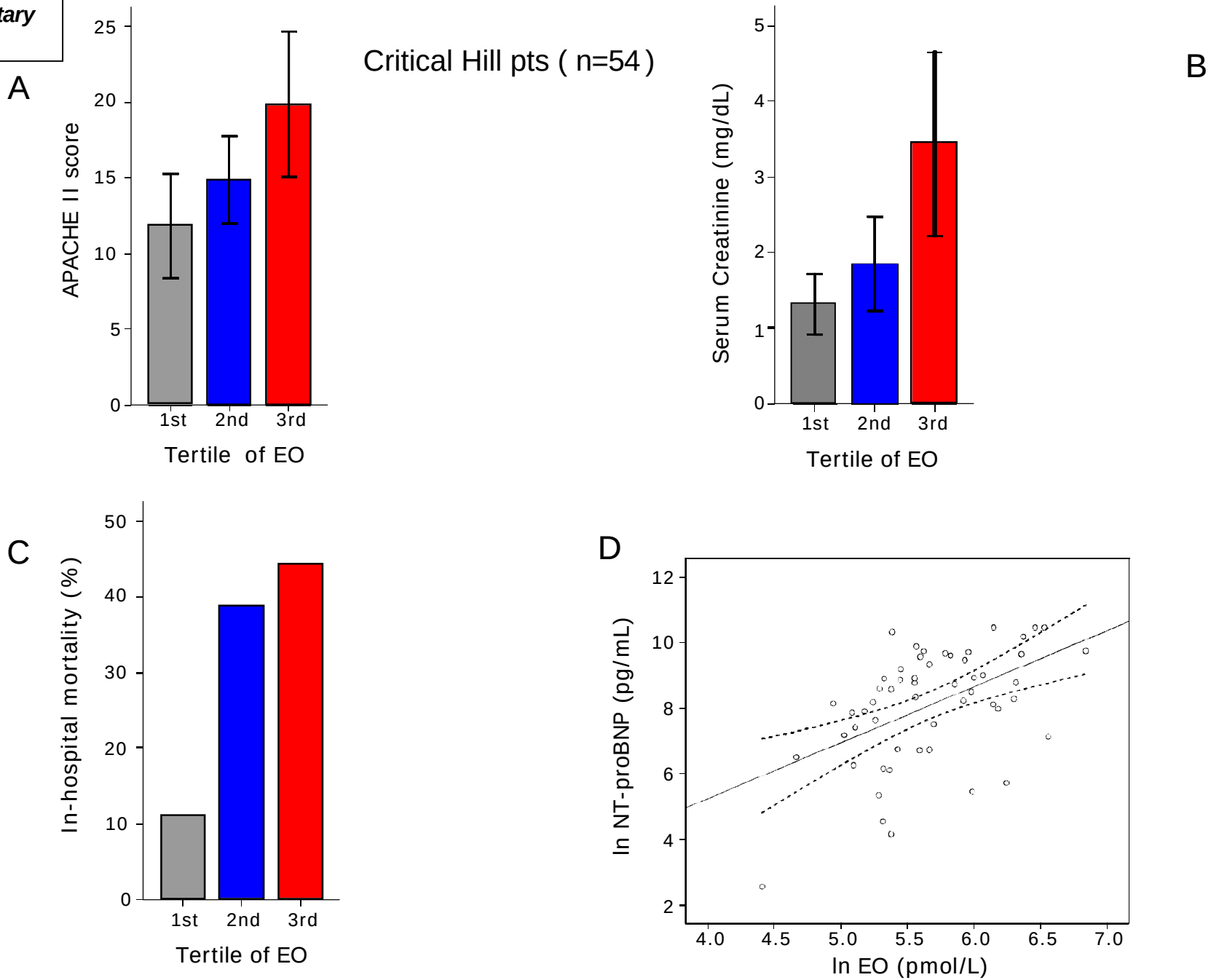
Age (yr)	57 ± 16
Male	57%
African American	56%
Mean arterial pressure (mmHg)	80 ± 21
Heart rate (bpm)	97 ± 25
EO (pmol/L))	270 (204 – 411)
NT-proBNP (pg/mL))	4565 (1151 – 13309)
Creatinine (mg/dL)	2.2 ± 1.9
Total bilirubin (mg/dL)	2.1 ± 3.7
APACHE score	15.5 ± 8.6
Ejection fraction (%)	52 ± 17
Left ventricular hypertrophy	24%
In-hospital death	32 %
Acute medical diagnosis	
Acute Kidney Injury	37%
Acute liver failure	17%
Gastrointestinal bleeding	19%
Pneumonia	44%
Acute respiratory distress syndrome	37%
Sepsis	43%
Shock	19%
ICU interventions	
Mechanical ventilation	63%
Intravenous vasopressors	22%
Transfusion of blood products	24%
Volume resuscitation	61%
Antibiotics	69%
Steroids	30%

Supplementary Table 6. Critical ill patients: clinical variables according to tertiles of endogenous ouabain (EO)

Variable	1st Tertile	2nd Tertile	3rd Tertile	P value
Age (years)	57 ± 11	59 ±18	57 ±16	0.9
Male	61%	56%	56%	0.9
African American	56%	61%	50%	0.6
Mean arterial pressure (mmHg)	87 ± 22	75 ± 19	78 ± 21	0.2
Heart rate (bpm)	97 ± 21	97 ± 27	98 ± 27	0.9
NT-proBNP (pg/mL; Median. IQR)	1494 (392 – 3497)	7290 (3282 – 15152)	7853 (3264 – 19501)	< 0.001
Creatinine (mg/dL)	1.3 ± 0.8	1.9 ± 1.3	3.4 ± 2.6	0.002
Total bilirubin (mg/dL)	0.6 ± 0.5	1.8 ± 2.3	3.9 ± 5.6	0.03
Ejection fraction (%)	56 ± 15	47 ± 16	55 ± 18.8	0.4
Left ventricular hypertrophy	22%	21%	30%	0.9
APACHE II score	11.8 ± 7.4	14.9 ± 6.2	19.9 ± 10.2	0.02
Death	11%	39%	44%	0.07
Acute medical diagnosis				
Acute Kidney Injury	11%	39%	61%	0.008
Acute liver failure	6%	0%	44%	0.001
Gastrointestinal bleeding	33%	11%	11%	0.1
Pneumonia	33%	50%	50%	0.5
Acute respiratory distress syndrome	29%	44%	39%	0.6
Sepsis	33%	50%	44%	0.6
Shock	11%	29%	17%	0.4
ICU interventions				
Mechanical ventilation	50%	72%	67%	0.4
Intravenous vasopressors	17%	33%	17%	0.4
Transfusion of blood products	28%	28%	17%	0.7
Volume resuscitation	67%	50%	67%	0.5
Antibiotics	56%	78%	72%	0.4
Steroids	28%	28%	33%	0.9
Medications prior to ICU admission				
ACEi or ARB	0%	17%	33%	0.03
β-blocker	22%	33%	56%	0.1
Diuretic	6%	22%	72%	< 0.001
Aldosterone antagonist	6%	11%	22%	0.3

Bronchodilator	22%	22%	22%	1.0
Prior Medical History				
Coronary artery disease	11%	22%	33%	0.3
Congestive heart failure	0%	11%	28%	0.04
Diabetes mellitus	22%	39%	33%	0.5
Hypertension	44%	50%	72%	0.2
Chronic obstructive pulmonary disease	11%	22%	22%	0.6
Chronic renal disease	17%	17%	22%	0.8

Supplementary Figure 1



Supplementary Figure 1: APACHE II score (A, p = 0.02) and, serum creatinine (B, p = 0.002) and in-hospital mortality (C, p = 0.07) grouped by tertile of endogenous ouabain. Scatter plot (D) of NT-proBNP (log converted) vs. endogenous ouabain (log converted). Endogenous ouabain (EO) is highly correlated with NT-proBNP.