**Study Protocol**

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**Title of the study:**

**“Sodium and Water homeostasis in children admitted with acute appendicitis: an observational prospective fluid balance cohort study”**

**1.0 Background and Rationale**

Hyponatremia, defined as plasma sodium (PNa) concentration <135 mmol/L, is the most common hospital-acquired electrolyte disturbance across all age groups (Feld L.G. *et al.* Pediatrics 2018; Winn Seay N. *et al.* Am. J. Kidney Dis. 2020). In majority of hospitalized hyponatremic patients, the decrease in PNa is primarily due to a disparity in electrolyte-free water intake and loss. In other words, hyponatremia is a metabolic disorder of water and not of sodium. The severity of symptoms is directly related to the acuteness and magnitude in the decrease of PNa.

Arginine vasopressin (AVP)-dependent trafficking of aquaporin-2 water channel in the renal collecting duct is crucial for the regulation of water homeostasis (Roche J.V. *et al.* Int. J. Mol. Sci. 2019). In the presence of AVP, vasopressin V2 receptors are activated stimulating translocation to and fusion of aquaporin 2–containing vesicles with the apical membrane of the principal cell, allowing water to flow into the cell, from which it exits into the hypertonic medullary interstitium via constitutive aquaporin-3 and aquaporin-4 channels in the basolateral membrane (Bichet D.G. *et al.* JASN 2006). Overall body fluid osmolality is regulated within a narrow range by water intake through thirst and water reabsorption in the kidney via the effect of AVP (Dunn F.L. *et al.* J. Clin. Invest. 1973)

There is a prevalent view that in hospitalized patients with high circulating plasma AVP levels the administration of hypotonic fluid therapy is invariably associated with the occurrence of hyponatremia (Jones DP. Pediatr Rev 2018). Consequently, the prescription of hypotonic fluid therapy to hospitalized patients is discouraged.

In a previous study, conducted in children admitted with acute appendicitis, we have observed that correction of hypovolemia with near-isotonic fluid solution followed by hypotonic maintenance intravenous fluid therapy was not consistently associated with post-operative hyponatremia, and that the degree of hyponatremia in patients that became hyponatremic was always mild (Lindestam U. *et al*. Ped. Res. 2019). More importantly, we noted that in patients with high circulating AVP levels, the degree of post-operative water retention declined significantly, as patients became euvolemic. This apparent escape from the antidiuretic effects of AVP attracted our attention and therefore is the focus of the proposed investigation.

Our assumption is that on admission most participants will have high circulating AVP levels. As a result of fluid therapy, we expect therefore a decrease of PNa at the end of surgery. The collection of new data has been planned based on an existing hypothesis, namely that we anticipate that some participants will show an escape from the antidiuretic effects of AVP (Lindestam U. *et al*. Ped. Res. 2019). To shed light on this phenomenon, we will evaluate the changes in PNa from hospital admission to the end of surgery in acutely ill normonatremic children with appendicitis and relate them to key hormones involved in sodium and water homeostasis, to how much free water is being excreted or reabsorbed, and to surrogate markers of AVP-dependent trafficking of aquaporin-2 water channel.

**2.0 Patients and Methods**

This a prospective observational cohort study that will be conducted in normonatremic children admitted with acute appendicitis at Sachs’ Children and Youth Hospital, Stockholm, Sweden.

Ethical approval was given by the Ethical Review Board in Stockholm (2019-03930). All parents will provide voluntary written informed consent for their children to participate in this study.

**2.1** *Inclusion criteria*

*i*). Male or female previously healthy children ≥10 and <15 years of age at the time of screening; *ii*). Admitted for suspected appendicitis.

*iii*). Informed consent obtained from parents and if applicable from the child.

**2.2** *Exclusion criteria*

*i*). Documented diagnosis of renal, endocrine, or metabolic disease; *ii*). PNa <135 mmol/L; *iii*). Having received intravenous fluid therapy before the admission at our hospital.

**3.0** *Primary objective*

*i*). The primary objective of this study is to assess mean PNa concentration directly after surgery in children with appendicitis in whom intravenous fluid therapy from the admission to the end of surgery has been administrated according to our *local recommendations*.

**3.1** *Secondary objective*

*i*). The occurrence of hyponatremia (PNa <135 mmol/L) directly after surgery.

**4.0** *Additional analysis*

In the current study, we will also evaluate whether mean plasma potassium (PK), plasma-chloride (PCl), plasma-albumin (PAlb), hemoglobin (Hb), plasma-creatinine (Pcr), serum-osmolality (SOsm), plasma-renin, plasma-aldosterone, plasma-AVP, and markers of nitric oxide metabolism (plasma-nitrate, and plasma-nitrite) and signaling (plasma cGMP) differ between admission, before induction of anesthesia, and directly after surgery. Also, we will investigate the mean changes in fractional excretion of Na from hospital admission to directly after surgery as well as the mean changes in urine osmolality (UOsm) and AVP-dependent trafficking of aquaporin-2 water channel from hospital admission to directly after surgery.

In addition, the association between mean changes in PNa from hospital admission to directly after surgery and the electrolyte-free water clearance calculated during the same period of observation both in the whole cohort study and separately in children with low and high circulating plasma AVP levels, will be evaluated. We plan to assess the association between the electrolyte-free water clearance calculated from hospital admission to directly after surgery and Uosm directly after surgery in the whole cohort study and separately in children with low and high circulating plasma AVP levels, respectively.

Finally, in case we confirm the escape from the renal actions of AVP, we will consider planning a post hoc analysis looking at post-translational modification of AVP using a combination of proteomics and mass spectrometry analysis of patient plasma samples as well as post-translational modification of aquaporin-2 water channel in the urine.

**5.0** *Fluid therapy*

Like our previous study, all participants will fast from admission to the end of surgery and will receive fluid therapy according to our *local recommendations*. Briefly, on admission, all patients will receive an intravenous infusion of 50 mL/kg of Ringer’s acetate solution (131 mmol/L sodium, 4 mmol/L potassium, 2 mmol/L magnesium, 110 mmol/L chloride, 30 mmol/L acetate; Fresenius Kabi®) over four hours. This infusion will be followed by a maintenance fluid and electrolyte therapy phase consisting of a hypotonic 0.46% normal sodium chloride (80 mmol/L sodium, 20 mmol/L potassium, 100 mmol/L chloride; extempore solution) in 5% glucose solution until the start of the surgery. At the maintenance stage, infusion rate will be decreased to 80% of normal maintenance fluid therapy. During surgery, fluids will be administered at anesthetist’s discretion. Anesthesia will be induced with fentanyl, propofol or thiopental, and rocuronium and maintained with sevoflurane.

**6.0** *Total number of patient*s

Since the primary endpoint is to evaluate the mean changes in PNa from hospital admission to the end of surgery, we calculate the sample size based on our previously published data (Lindestam U. *et al*. Ped. Res. 2019). We observed that the mean change between PNa on admission and at the end of surgery was 3.55 mmol/L (*n* = 52; Std. Error 0.33, lower bound 2.7 and upper bound 4.3 mmol/L; PNa on admission was 138.1 mmol/L (SD ± 2.77) and at the end of surgery was 134.6 mmol/L (SD ± 2.1), respectively). The median (interquartile range) time lapse from admission until the end of surgery was 13 (9.5-19.8) hours. Based on this premise, we calculated the sample size for the current study using a power of 90% to detect a 3.5 mmol/L mean change in PNa with an assumed standard deviation of 3.0 mmol/L, and with a two-sided controlled at the type-I error rate of 0.05. The results show that 25 participants are needed to meet the power goal allowing for approximately 10% unexpected dropouts. The sample size calculation was performed using nQuery Advisor®.7.0.

**7.0** *Laboratory analysis*

On admission, before the start of fluid therapy (baseline), at induction of anesthesia, and directly after surgery PNa, PK, PCl, venous blood gas, PAlb, Hb, Pcr, SOsm, plasma-renin, plasma-aldosterone, plasma-AVP, plasma-nitrate, plasma-nitrite, and plasma cGMP will be investigated. A spot urine sample will be obtained at baseline as well as directly after surgery and will be analyzed for UOsm, Ucr, urine sodium (UNa), AVP-dependent trafficking of aquaporin-2 water channel, and noradrenaline. In addition, all urine will be carefully collected from admission until the end of surgery, the collection recorded and measured volumetrically, and analyzed for UOsm, Ucr, UNa, urine potassium (UK), and urine chloride (UCl), respectively.

Routine laboratory tests will be performed according to accredited hospital clinical laboratory procedure at the Department of Laboratory Medicine Södersjukhuset, Stockholm, Sweden. Plasma-sodium will be assessed by indirect ion-selective electrode method. Plasma-renin, plasma-aldosterone, and AVP will be analyzed at Department of Cardiovascular and Renal Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark. AVP-dependent trafficking of aquaporin-2 water channel will be determined at the Department of Biochemistry and structural Biology, Lund University, Lund, Sweden. Plasma-nitrate, plasma-nitrite, and plasma cGMP will be analyzed at the Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden. Post-translational modification of AVP will be performed at the Department of Biomedicine and Clinical Sciences, Linköping University, Linköping, Sweden. Noradrenalin levels will be measured in urine using enzyme-link immunosorbent assay (ELISA, IBL, Hamburg) at Sophiahemmet University. Aliquots of urine will be acidified with HCl as preservative and stored at -20˚ until use. Samples will be analyzed according to the manufacturer´s protocol.

**8.0** *Equations:*

*i*). The fractional excretion of sodium (FENa) will be calculated from a spot urine sample taken at baseline and at the end of surgery, as follows:

FENa = [(UNa ∕ Ucr) ∕ (PNa ∕ Pcr)] 100

*ii*). Electrolyte osmolar clearance (E-COsm), or “effective osmolal clearance”, which refers to the clearance of only effective osmoles, *i.e.*, Na, K and their accompanying anions will be calculated as follows (Shimizu K. *et al.* Nephron 2002):

E-COsm (mL/min) = UNa + K × V ∕ PNa

*iii*). Electrolyte-free water clearance (E-CH₂O), which is the most direct method to determine whether free water is reabsorbed from or added to the tubular fluid during urine concentration, will be calculated as follows:

E-CH₂O (mL/min) = V - E-COsm

V, urinary flow rate (mL/min)

**9.0** *Statistical analysis:*

Statistical analyses will be performed using IBM SPSS Statistics for Windows version 24 (IBM Corp, Armonk, NY). All continuous variables will be presented as medians and interquartile ranges (IQR), unless otherwise stated. Paired t-test will be carried out to compare individual differences between admission and at the end of surgery, Pearson correlation coefficient will be used to measure association between two variables, Fisher’s exact test will be used to compare dichotomous variables, and analysis of variance (ANOVA) followed by the post-hoc Bonferroni test, will be performed for multiple pair-wise comparisons, *i.e.*, between admission, before induction and at the end of surgery. Two-tailed p values less than 0.05 will be considered statistically significant.