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Colonocyte NHE3 protein expression in acute heart failure

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ABBREVIATIONS

HF: Heart failure

LVEF: Left ventricular ejection fraction

HFpEF: Heart failure with preserved ejection fraction

HFrEF: Heart failure with reduced ejection fraction

HFmrEF: Heart failure with mid-range ejection fraction

RHF: Right heart failure

RVD: Right ventricular dysfunction

AHF: Acute heart failure

CVP: Central venous pressure

RAAS: Renin-angiotensin-aldosterone system

NHE: Sodium/hydrogen exchanger 3

PMH = past medical history

GI = gastrointestinal

BID = bowel intestinal disease

PAH = pulmonary arterial hypertension

GFR: Glomerular filtration rate

MFI: Median fluorescence intensity

S/N: Signal to noise ratio

ACEI: angiotensin-converting enzyme inhibitor

ARB = angiotensin receptor blocker

BACKGROUND

A. Heart failure

Heart failure (HF) is caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest or during stress. It leads to a clinical syndrome characterized by symptoms, like dyspnea and fatigue and signs such as elevated jugular venous pressure and peripheral oedema.[1] It is a major public health concern, affecting 1-3% of the adult population in developed countries.[2] The prevalence of HF keeps rising with the aging of the population, and is currently estimated at 26 million people worldwide, and 6.5 million adults in the United States.[3] It is is one of the leading cause of morbidity and mortality, with a an absolute mortality rate at 50% within 5 years of diagnosis. HF is the primary diagnosis in > 1 million hospitalization annually, in the United States.[4]

According to the European Society of cardiology (ESC), HF can be classified into 3 categories based on the left ventricular ejection fraction (LVEF):

- HF with reduced EF (HFrEF), with a LVEF < 40%, previously referred as "systolic HF"
- HF with preserved EF (HFpEF), with LVEF > 50%, previously referred as "diastolic HF"
- HF with mid-range EF (HFmrEF) with a LVEF between 40 and 50%

Despite similar symptoms and signs between those 3 categories, pathophysiology, co-morbidities and response to therapy are different. Among HF patients, approximately half have HFrEF and the other half have HFpEF or HFmrEF.[5,6]

Right HF (RHF) is a clinical diagnosis with signs and symptoms of systemic congestion in combination with structural and/or functional abnormalities of the right heart. In left-sided HF, right ventricular dysfunction (RVD) is recognized as a major prognostic factor. Its prevalence is about 60% in HFrEF[7] and 30 to 50% in HFpEF [8] depending on the modality used to assess RV function. In both HFrEF and HFpEF, RVD has been found to be associated with an increased risk of mortality and HF hospitalization.

Acute HF (AHF) refers to rapid onset of new or worsening of symptoms and/or signs of HF. It includes patients presenting for the first time (de novo AHF), and more often those with acute decompensation of chronic HF. Initial diagnosis is based on prior cardiovascular history and potential cardiac and non-cardiac precipitants, assessments of signs and symptoms reflecting fluid retention (pulmonary and systemic congestion) and/or reduced cardiac output (peripheral hypoperfusion). The diagnosis is further confirmed by additional investigations including ECG, chest X-ray, laboratory assessment (elevation of biomarkers such as BNP>35pg/ml or NT-pro BNP>125pg/ml) and echocardiography. In acute HF, mortality during admission is high, ranging from 5 to 15%. Of the discharged patients, a further 10-15% will die within 6-12 weeks and one-third will be re-hospitalized for all cause, with a 1-month readmission rate of 25%.[9,10]

B. The importance of venous congestion

1. *Definition*

Congestion in heart failure is defined as signs and symptoms of extracellular fluid accumulation that result in increased cardiac filling pressures.[11] The gold standard for diagnosing congestion in heart failure is cardiac catheterization to measure right atrial pressure and pulmonary wedge capillary pressure (PCWP). However, the invasive nature of catheterization limits its routine in clinical practice, and congestion is usually detected by signs and symptoms of systemic congestion (peripheral edema, jugular venous pulsation, hepatomegaly) and pulmonary congestion (dyspnea, end-inspiratory crackles), in combination with non-invasive techniques (chest X-ray, echocardiography, natriuretic peptides).

2. *Pathophysiology*

Although pathogenesis of venous congestion is still incompletely understood, neurohormonal activation of both the sympathetic nervous system and the renin-angiotensin-aldosterone system (RAAS) plays a major role. Initially beneficial in maintaining cardiac output through increased heart rate and contractility, and peripheral arterial vasocontriction to maintain tissue perfusion, the chronic activation of these systems ends up being maladaptative and results in worsening HF. Notably, besides increasing heart rate, contractility, and systemic vascular resistance, these neurohormonal systems also increase preload by augmenting fluid retention at the level of the kidney. Angiotensin II stimulates activity of sodium/hydrogen exchanger 3 (NHE3) and sodium/potassium ATPase (NaK ATPase) in the proximal tubule, leading to increased sodium resorption. It also promotes the release of aldosterone resulting

in increased sodium resorption in the distal tubule and collecting duct, through stimulation of epithelial sodium channel (ENaC) and Na-K ATPase. Finally, angiotensin II provokes the release of vasopressin, resulting in water retention. All of these mechanisms contribute to the progressive volume expansion observed during transition from chronic to acute decompensated HF.

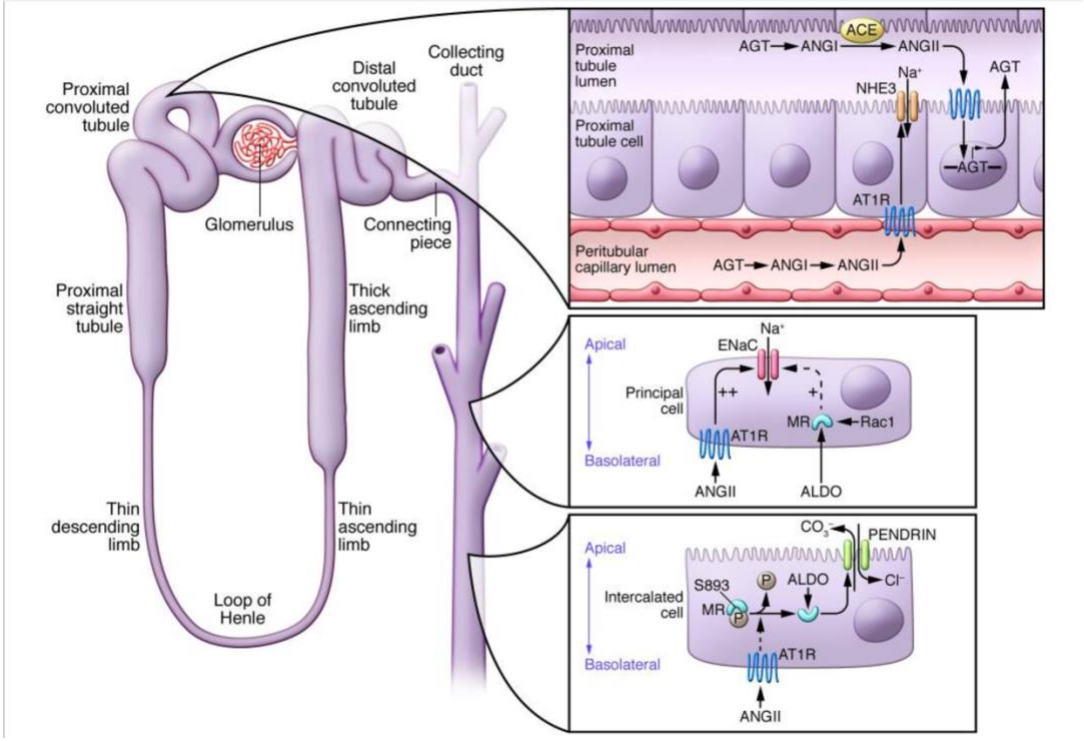

Figure 1. Role of RAAS within the kidney.

Ag II, derived from angiotensinogen generated in the liver, binds to AT1 receptors in the proximal tubule, influencing the synthesis and activity of NHE3. In the collecting duct, AgII and aldosterone increase sodium absorption, through stimulation of ENaC. From Coffman et al. *J Clin Invest.* Jun 2014.[12]

3. <u>Consequences of venous congestion</u>

The vast majority of acute heart failure hospitalization are driven by signs and symptoms of venous congestion, whereas only a minority of patients present acutely with signs of low perfusion. Clinical congestion has shown to be an important predictor of HF hospitalization and mortality. A post-hoc analysis of the Studies of Left

Ventricular Dysfunction (SOLVD) prevention trial showed that signs of congestion (elevated jugular venous pressure, and S3) in chronic HF were associated with an increased risk of HF hospitalization and all-cause death.[13]

Moreover, venous congestion is involved in organ dysfunction. It's a major determinant of the cardiorenal syndrome. In 2009, Mullens et al. showed that CVP, rather than impairment of cardiac output, was most closely associated with worsening renal function in patients with advanced decompensated HF.[14] The increase in central venous pressure (CVP) due to HF leads to increased renal intersticial pressure, attenuating the transglomerular pressure gradient, causing a reduction in the glomerular filtration rate (GFR) and tubular lesions.[15]

Venous congestion is also a determinant of HF-induced hepatic and gastro-intestinal dysfunction. Markers of cholestasis are independently associated with HF hospitalization and mortality among patients with HF.[16] Moreover, elevated right atrial pressure, in combination with right ventricular dysfunction in patients with chronic HF has shown to be strongly associated with cachexia, with patients having a higher prevalence of post-prandial fullness, appetite loss and abdominal discomfort.[17] Compared with control patients, chronic HF patients exhibit an increased bowel wall thickness, increased intestinal permeability and increased mucus-adherent bacterial growth[18] suggesting that venous congestion may contribute to chronic inflammation in HF patients, as the gut becomes more permeable and susceptible to bacterial translocation.

4. *Management of venous congestion*

Achieving decongestion in AHF is crucial. Thus, clinical congestion at discharge is a strong predictor of poor outcome and readmission. In the Evaluation Study of Congestive Heart Failure (ESCAPE), patients who lost more weight and had a greater degree of hemoconcentration at discharge, had a lower 180-day mortality, despite significant worsening of renal function.[19]

Decongestive therapies are mainly based on diuretics, with loop diuretics being the most widely used. However, over time, a lot of patients experience diuretic resistance, defined as an impaired sensitivity to diuretics resulting in reduced natriuresis and diuresis limiting the possibility to achieve euvolaemia.[11] The pathophysiology of diuretic resistance is multifactorial (RAAS activation, nephron remodeling, pre-existing renal function alterations, disrupted pharmacokinetics and dynamics of diuretics). Therefore, there is a major unmet need for a better understanding of the mechanisms of congestion to help develop new therapeutics in the future.

C. Role of the gastrointestinal tract in water and sodium resorption: Another determinant of venous congestion?

1. *Pathophysiology of water and electrolytes absorption*

The colon (large intestine) extends from the terminal ileum to the anal canal. Its wall consists of four layers: the mucosa, the submucosa, the circular muscle and the longitudinal muscle. The mucosa is composed by a multitude of crypts on its surface, consisting of 3 different types of cells including colonocytes. The colon plays an important role in maintaining salt, and fluid balance: under normal physiological

conditions, approximately 1.5L of fluid enters the colon each day, but only 100-200mL are excreted. The mechanisms responsible for sodium absorption in the colon are secondary to transport proteins, mainly sodium/hydrogen exchangers (NHE) located on the apical membrane of the colonocytes. NHE use the electrochemical concentration gradient developed across the plasma membrane by the basolateral Na/K ATPase pump which pumps sodium from inside the colonocyte against a large concentration gradient into the intercellular space. The hypertonic fluid within the intercellular space draws water passively into the mucosa from the lumen.[20]

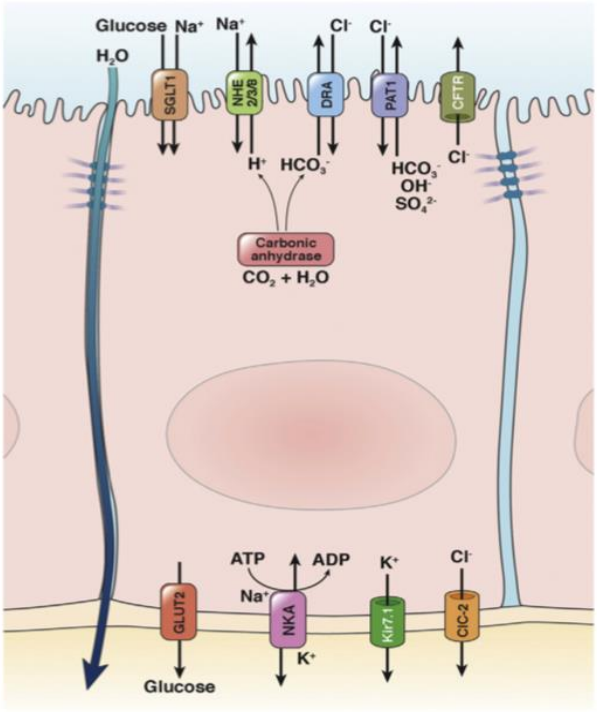

Figure 2. Ions transporters and channels involved in Na+ and Cl- transport in epithelial cells

From Gurney & al. Pathophysiology of Intestinal Na(+)/H(+) exchange, 2017.[21]

2. *Sodium-Hydrogen Exchanger 3 (NHE3)*

NHE3 appears to be the main sodium channel expressed in the colon. On the nine members of the NHE family identified to date, NHE2, NHE3 and NHE8 are the ones localized in the apical membrane of the intestinal tract, playing a role in intestinal sodium absorption. The importance of NHE3 in sodium absorption and water homeostasis in the intestine became evident from studies of NHE3 knockout mice, which develop diarrhea, low blood pressure and metabolic acidosis in the absence of NHE3.[22] NHE2 knockout mice, on the contrary, exhibited no apparent abnormality in intestinal sodium absorption. NHE8 has shown to play a role in the early stages of development. NHE3 is implicated in regulating cell volume and nutrient absorption, but also control of the intracellular pH. It is located at the plasma membrane and in the endosomal compartment (intracellular NHE3). It is closely regulated, and its downregulation has proved to play a role in inflammatory bowel diseases and infectious diarrheas.[21]

3. *How could NHE3 be upregulated in HF?*

a) Neurohormonal activation

Several pathways have proved, in *in vitro* and *in vivo* studies, to upregulate intestinal NHE3 expression and activity. In 2009, Musch et al. investigated the action of ATII on sodium transport in human intestinal epithelial Caco2BBE cells, and found that ATII increased expression and activity of apical NHE3.[23] Moreover, they found that after 1-2h of treatment, apical membrane NHE3 expression increased without any change in total protein abundance suggesting translational modification such as trafficking from endosomal compartment to the apical membrane. After 2 hours of

treatment, NHE3 mRNA increased, suggesting increased gene transcription. Similarly, experimental hyperaldosteronism has shown to cause an increase in intestinal sodium absorption in rats, by increasing NHE3 expression and activity in the proximal colon.[24] Finally, catecholamine (noradrenaline) may also upregulate intestinal NHE3, as several studies demonstrated its role in increasing NHE3 expression and activity in the proximal tubule of the kidney[25].

As described previously, neurohormonal activation of the sympathetic system and the RAAS in HF patients leads to increased ATII, aldosterone and catecholamine. It could enhance intestinal NHE3 expression, leading to increased sodium and water absorption.

b) Regional metabolic acidosis

The fact that one of the major pathway for sodium absorption in the colon is mediated by NHE3, a Na+/H+ exchanger suggests a possible link between acid-base balance and sodium absorption. Therefore, several studies showed an increase in NHE3 protein expression and activity in rats with metabolic acidosis.[26,27]

In HF patients, abdominal hemodynamics can be modified through different mechanisms. Elevated splanchnic venous pressure coupled with decreased splanchnic arterial blood flow reduces the arterial-venous pressure gradient across the intestinal capillary network. The microstructure of the intestinal villus forms a plexus, an ideal structure to optimize nutrient absorption, but also susceptible to shunting of oxygenated blood through the base of the villus, putting the villus tip at risk of a relative ischemia.[28] This relative ischemia could promote anaerobic metabolism, leading to

regional acidosis, and promoting intestinal NHE3 upregulation to regulate the intracellular acid-base balance. However, increased NHE3 activity would also result in (1) absorption of sodium and water, increasing volume overload, and (2) extrusion of H+ into the gut lumen. This extrusion of H+ into the gut lumen could be responsible for the dysbiosis observed in the HF population.[29,30]

Systemic, and intestinal congestion occurring primarily in HF with RVD, this upregulation mechanism might be even more important in HF patients with worse RV function, as intestinal congestion would increase intestinal ischemia and intracellular acidosis in the colon. Thus, intestinal NHE3 activity could be higher in HF patients with RVD.

D. Objectives

The objective of the present study was to investigate the expression of cell surface and intracellular NHE3 protein in isolated colonocytes from hospitalized HF patients, compared to hospitalized non-HF controls. Secondly, we aimed to investigate the relationship between NHE3 expression and RV function.

MATERIALS AND METHODS

A. Study population

The study is a single-center, prospective, observational study. From April, 2017 to August, 2018, patients referred to the Northwestern Memorial Hospital (Chicago, Illinois, USA) with a primary diagnosis of ADHF were screened. Patients with a past medical history (PMH) of gastrointestinal (GI) disease, including gastrointestinal cancer, bowel inflammatory disease (BID), enteritis or colitis and history of surgical bowel resection were excluded, as well as patients with a history of cirrhosis, heart transplant or ventricular assist device, pulmonary arterial hypertension (PAH) and severe renal failure (glomerular filtration rate [GFR] < 15 mL/min/1.73 m^2), including patients on dialysis. Patients hospitalized for another reason, without a history of HF (and no evidence of current HF) were screened for inclusion into the control group. Exclusion criteria for controls were identical to HF patients. The present study was approved by the Northwestern University Institutional Review Board. Informed consent was obtained from all patients prior to their participation in the study.

B. Outcomes

The primary outcome was evaluation of cell surface and intracellular NHE3 expression in exfoliated colonocytes, measured with flow cytometry. The secondary outcome involved study of the correlation between NHE3 expression and indicators of right ventricular (RV) structure and function (tricuspid annular plane systolic excursion [TAPSE], RV basal free wall tissue Doppler [s'] velocity, and RV basal diameter).

C. Measurements

Demographics, clinical comorbidities, medication date, vital signs, and blood laboratory test data were obtained from chart review.

1. Echocardiography

A standardized two-dimensional and Doppler echocardiography [including M-Mode, 2 dimensional, spectral, color flow and tissue Doppler] was performed by Registered Diagnostic Cardiac Sonographers using a Philips IE-33 or a Vivid 7 ulstrasound machine (GE healthcare) interfaced with a standard 2.5-3.5 MHz phased-array probe. Echocardiograms were analyzed and interpreted using EchoPAC Software (GE Healthcare). The values of all 2DE echocardiographic parameters were calculated according to the current American Society of Echocardiography (ASE) recommendations.[31] Left ventricular ejection fraction (LVEF) was estimated by using the biplane method. Pulsed Doppler measurements included the transmitral early diastolic peak flow velocity (E), and the ratio between E and early LV diastolic relaxation velocity (tissue Doppler-derived e' velocity, average septal and lateral mitral annulus; E/e' average). The RV diameters were measured in the 4-chamber apical view (basal, mid cavity and longitudinal RV diameter). RV function was assessed by measuring the fractional area change (FAC) from the apical 4-chamber view < 35%, TAPSE, and RV s'. Right atrial area (RAA) was obtained in the 4-chamber apical view. Finally, we estimated right atrial pressure (based on size and collapsibility of the inferior vena cava), and pulmonary artery systolic pressure (from the combination of the peak tricuspid regurgitation velocity and the estimated right atrial pressure), as described previously.[32]

2. *Isolation of exfoliated colonocytes*

Exfoliated colonocytes were isolated using SCSR™ (Somatic cell sampling and recovery) fecal cell isolation kit (Noninvasive Technologies, Elkridge, Maryland, USA), a noninvasive technique for isolation of colonocytes, used in colorectal cancer and inflammatory bowel disease to study biomarkers. Approximately 1g of stool was collected from the patient, and placed in a screw-capped collection vial containing 15mL of a nontoxic transport medium (SCSR-T). After a preliminary filtration through a 330μm nylon mesh to remove large particulate matter of dietary origin, the filtrate was passed through a second filter (40μm) into a new 50mL centrifuge tube and underlaid with 10mL of the cushion medium (SCSR-C). The tube was then centrifuged for 10 minutes at 20°C at 200g. The colonocytes were aspirated from the interface between the two layers, and washed by adding cold PBS pH 7.2 adjusting the total volume to 30mL.The tubes were then centrifuged for 10 minutes at 4°C at 2000g. Washing with PBS followed by centrifugation was repeated three times, after which 100μm of the pellet obtained was re-suspended in 800μm of cold PBS pH 7.2 and 100μm of freezing medium (DMEM base media, FBS 30%, DMSO 10%). The aliquots were subsequently stored at -80°C.

3. *Flow cytometry*

Following thawing, cell suspensions were washed twice in staining buffer (PBS + 2% BSA) and stained for cell surface markers with primary anti-NHE3 antibodies (1μL of primary anti-NHE3 antibody, in 100μL of single cell suspension). Cells were incubated at room temperature for 30 minutes, washed in and then stained for secondary antibodies with conjugate for 30 minutes at room temperature in the dark, washed and resuspended in staining buffer. For intracellular staining, cell suspensions

were washed twice in staining buffer, and Formaldehyde was added to each tube (65μL of formaldehyde in 100μL of single cell suspension) for permabilization. Cells were incubated at room temperature for 10 minutes, and washed before being incubated with 0.1% Triton-X solution in PBS (2mL) for 10 minutes. Cells were then washed, and stained for primary anti-NHE3 antibodies and secondary antibodies conjugate following the same protocol as above. Cells were first gated by forward-scatter versus side-scatter area plot (Figure 3A), and singlets were then gated using forward-scatter area versus forward-scatter height. Cells were analyzed on a BD LRSFortessa and events collected were analyzed using FlowJo Version 9.6.4 software (Tree Star).

D. Statistical analysis

We compared HF patients and control patients. Continuous data were expressed as means (± standard deviation [SD]) or medians (25^{th}, 75th percentile [IQR]) based on the distribution, and categorical variables as counts and percentages. Comparison of continuous variables among groups was performed using t-test or Mann-Whitney-U test (when indicated), and categorical variables were compared using the chi-square or Fisher's exact test, as appropriate. Normality of distribution was assessed using the Kolmogorov Smirnov test. To assess for potential cofounding, we used a multivariable linear regression analyses, with NHE3 expression as the dependent variable, HF status as the primary independent variable, and adjusted for covariates that have been implicated in NHE3 expression regulation (age, diabetes mellitus, atrial fibrillation, coronary artery disease, hypertension, ACE-inhibitor or ARB, beta-blocker, glucocorticoids, and GFR). The Spearman correlation test was used to examine the

relationship between NHE3 expression and RV parameters. We also used a linear regression to examine the association between RV parameters and NHE3 expression. Differences were considered statistically significant at p-value < 0.05. All statistical analysis was conducted with STATA 12.0 software (Stata Corp.; College Station, Texas, USA).

RESULTS

A. Patients characteristics

Of the 39 HF patients, and 25 controls enrolled in the study between April, 2017 and August 2018, NHE3 expression could be assessed on 24 HF patients, and 10 controls (Figure 3). HF patients were 73.4 ± 13 years old, mainly Caucasians (75%), with a majority of patients in NYHA class ≥ 3 (77%). Median LVEF was 31.8% (27.2, 51.0), median TAPSE was 13.5mm (9.9, 17.5) and median BNP was 477 pg/mL (287.0, 1265.0). Patients with HF did not significantly differ from controls regarding age, sex, race/ethnicity, diabetes, hypertension, smoking status, and renal function, but were more likely to have atrial fibrillation (p=0.020) and coronary artery disease (p=0.024) (Table 1).

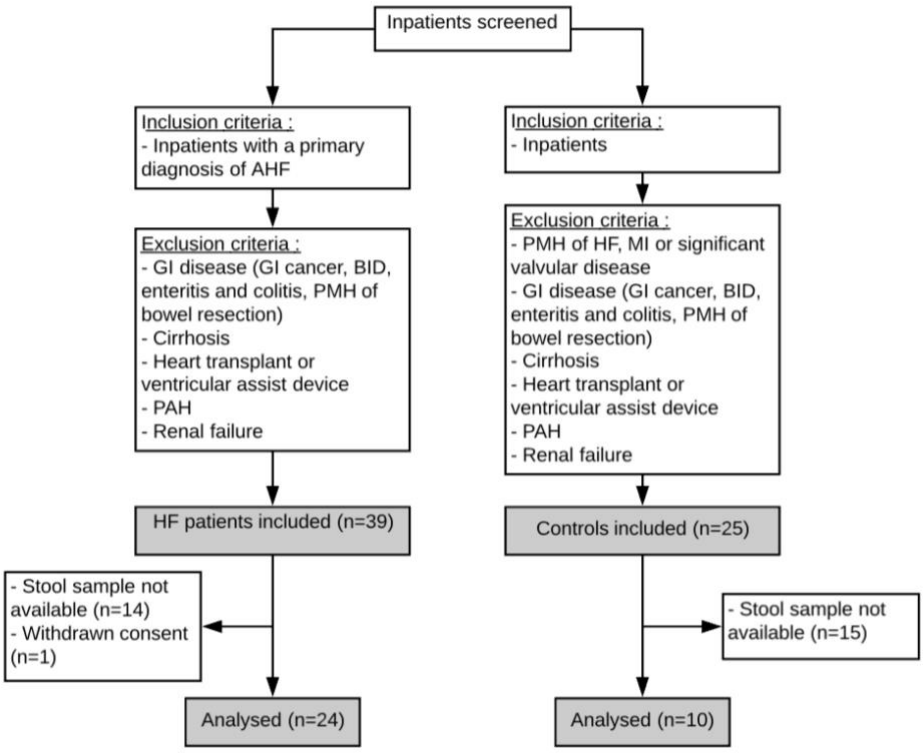

Figure 3. Flowchart

Table 1: Baseline characteristics

	HF (n=24)	Controls (n=10)	p-value
Age	73.4 ±13.0	66.0 ±12.9	0.14
Male	17 (71%)	4 (40%)	0.13
Caucasian	18 (75%)	9 (90%)	0.64
Weight, kg	93.8 ± 33.5	79.0 ± 21.5	0.21
Body surface area, m^2	2.1 ± 0.4	1.9 ± 0.3	0.21
Hypertension	16 (67%)	5 (50%)	0.45
Hyperlipidemia	15 (62%)	4 (40%)	0.28
Diabetes mellitus	9 (38%)	1 (10%)	0.22
History of atrial fibrillation	14 (58%)	1 (10%)	0.020
Coronary artery disease	13 (54%)	1 (10%)	0.024
Smoking	1 (4%)	2 (20%)	0.20
Obstructive sleep apnea	6 (25%)	2 (20%)	1.00
NYHA functional class ≥3	17 (77%)	0 (0%)	<0.001
Systolic BP	118.2 ± 18.4	132.3 ± 14.6	0.039
Diastolic BP	71.4 ± 22.5	68.6 ± 8.0	0.70
Heart rate	76.9 ± 15.2	71.8 ± 14.4	0.38
Baseline medication			
β-blockers	16 (67%)	4 (40%)	0.25
ACEI or ARB	14 (58%)	3 (30%)	0.26
MRA	11 (46%)	1 (10%)	0.061
Loop diuretics	18 (75%)	1 (10%)	0.001
Glucocorticoids	0 (0%)	2 (20%)	0.080
Laboratory parameters			
Sodium, mEq/l	139.1 ± 3.8	140.1 ± 2.3	0.45
Glucose, mg/dl	114.6 ± 36.2	121.0 ± 24.0	0.61
Hemoglobin, g/dl	11.7 ± 1.8	12.3 ± 2.8	0.46
Creatinine, mg/dl	1.3 ± 0.7	1.0 ± 0.4	0.23
GFR, ml/min/1.73 m^2	61.9 ± 29.2	70.7 ± 22.6	0.40
BNP, pg/ml	477.0 (287.0, 1265.0)	72.0 (40.0, 76.0)	0.006
Echocardiography parameters			
LVEF, %	31.8 (27.2, 51.0)	60.3 (57.6, 66.1)	0.001
LVEDV index, ml/m^2	53.1 (39.9, 69.2)	32.0 (23.2, 43.1)	0.006
LVESV index, ml/m^2	30.3 (23.3, 45.1)	11.1 (9.3, 13.7)	<0.001
Septal wall thickness, mm	11.7 (3.3)	10.7 (2.8)	0.47
Cardiac output index, L/min/m^2	1.8 (1.5, 2.2)	3.4 (2.4, 5.0)	0.004
E/A ratio	2.5 (1.6, 2.9)	1.0 (0.8, 1.2)	0.001
E/e' average	17.6 (13.4, 24.9)	9.8 (9.2, 19.2)	0.11
LA volume index, ml/m^2	38.6 (25.6, 43.0)	23.7 (18.3, 26.1)	0.004
TAPSE, mm	13.5 (9.9, 17.5)	19.7 (18.0, 21.0)	0.004
RV s' velocity, cm/sec	7.7 (5.9, 10.0)	12.4 (12.0, 14.0)	<0.001
RV FAC, %	24.6 (18.7, 30.5)	47.4 (41.1, 52.6)	0.003
RV basal diameter, mm	38.0 (35.0, 41.2)	30.5 (28.3, 36.30	0.008
IVC maximal diameter	22.0 (21.0, 26.0)	13.5 (10.0, 17.9)	<0.001
Estimated RA pressure	8.0 (3.0, 15.0)	3.0 (3.0,3.0)	0.008
Estimated PA pressure	48.9 (37.8, 59.1)	26 (23.1, 32.2)	0.013

Values are n (%), mean ± SD or medians (25^{th}, 75^{th}), ACEI = angiotensin-converting enzyme inhibitor; ARB = angiotensin receptor blocker; MRA = mineralocorticoid receptor antagonist; GFR = glomerular filtration rate; BNP = brain natriuretic peptide; LVEF = left ventricular ejection fraction; LVEDV = left ventricular end diastolic volume; LVESV = left ventricular end systolic volume; LA = left atrium; TAPSE = Tricuspid annular plane systolic excursion; FAC = fractional area change; IVC = inferior vena cava, RA = right atrial, PA = pulmonary artery.

B. Cell surface and intracellular NHE3 protein expression

1. Cells identification

Distribution of cells according to their size (forward scattered light [FSC]) and structure (side scattered light [SSC]) is presented in Figure 3A. One cell type was predominant, and 90% of the cells were gated. After that first gating, events that could represent more than 1 cell (doublets) were excluded from the analysis (Figure 3B). Staining the remaining cells with a CD45 antibody revealed that less than 1% of the cells were hematopoietic cells. For each subject studied, control of fluorescence was done with unstained cells (Figure 3C) (cells were incubated with secondary antibodies, in the absence of primary antibodies) used as negative controls. In order to exclude the background fluorescence (cellular autofluorescence, and nonspecific staining), signal to noise ratio (S/N) was measured by dividing the median fluorescence intensity (MFI) of the positive cells by that of the negative controls. In the HF population, as well as in the control group, almost all cells expressed NHE3 protein, although there were significant differences between the 2 groups: (median [IQR] cell surface NHE3 positivity = 100 (99.7,100)% (HF), vs 95.6 (93.4,99.5)% (controls), $p<0.001$; Intracellular NHE3 positivity = 100 (100, 100)% (HF) vs 99.4 (99.2, 99.9)% (controls), $p<0.001$).

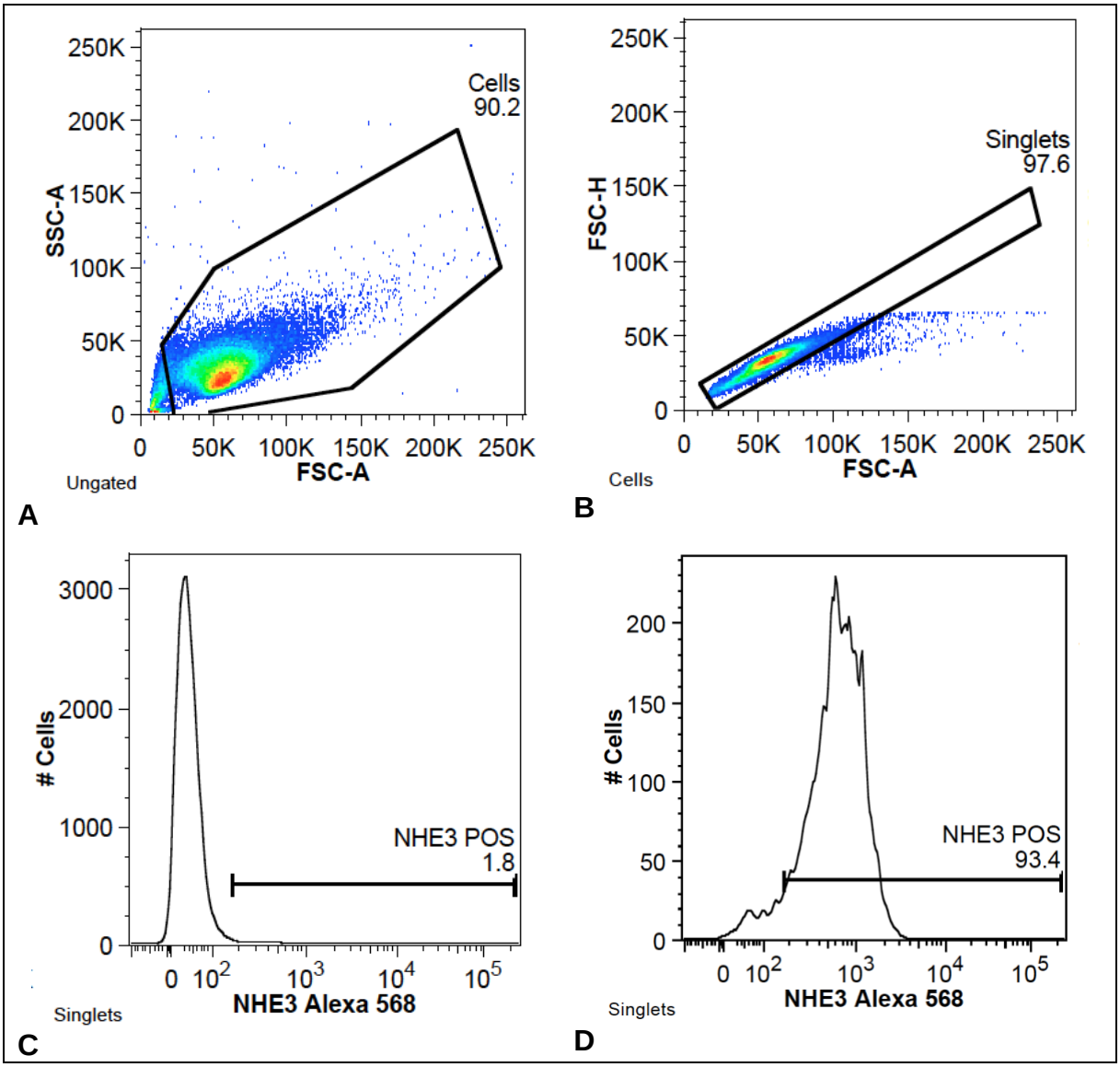

Figure 3. Flow cytometry

(A) Density plot representing the distribution of cells in the light scatter based on size (FSC-A) and intracellular composition (SSC-A) (B) Density plot representing the distribution of cells based on their size (FSC-Height, and FSC-Area): Exclusion of events that could represent more than 1 cell (C) Fluorescence of unstained cells (negative control) (D) Fluoresence of stained cells (positive cells).

2. *NHE3 expression: signal to noise (S/N) ratio*

Cell surface, and intracellular NHE3 expression were assessed by S/N (signal-to-noise ratio, as described above). As shown in Figure 4, both cell surface and intracellular NHE3 expression were significantly increased in HF patients compared to

controls: median (IQR) S/N = 79.4 (29.8, 165.4) vs 21.3 (17.8, 31.1), p<0.001 and 112.0 (58.6, 169.3) vs 10.1 (7.7, 13.6), p<0.001, respectively. On a linear regression analysis, both cell surface and intracellular S/N were significantly higher in HF patients, compared to control even after adjusting for age, hypertension, atrial fibrillation, coronary artery disease, ACEI or ARB, beta-blocker, corticosteroids and glomerular filtration rate (GFR) (table 2).

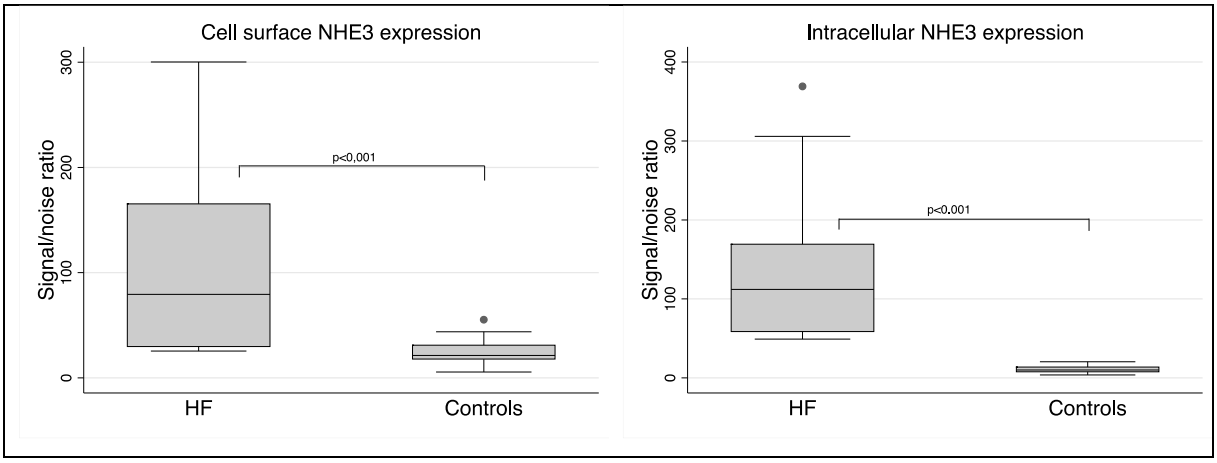

Figure 4. Cell surface and intracellular NHE3 expression, assessed by the signal to noise ratio of the fluorescence of NHE3 based on flow cytometry.

Table 2: Association of heart failure status with colonocyte NHE3 expression on linear regression analysis

NHE3	**Unadjusted**		**Adjusted***	
	β-coefficient (95% CI)	p-value	β-coefficient (95% CI)	p-value
Cell surface S/N	74 (24.4, 124.4)	0.005	93.2 (21.9,164.4)	0.013
Intracellular S/N	127 (69.9, 184,8)	<0.001	158.9 (83.2,234.7)	<0.001

*Adjusted for age, diabetes mellitus, atrial fibrillation, coronary artery disease, hypertension, ACE-inhibitor or ARB, beta-blocker, glucocorticoids, and GFR.
CI = confidence interval; S/N = signal to noise ratio on flow cytometry

C. Relationship between NHE3 expression and RV parameters

Both cell surface, and intracellular NHE3 expression showed a strong correlation with RV functional and size parameters, such as TAPSE (r_s=-0.60, p<0.001, and r_s=-0.56, p=0.002 respectively), RV s' velocity (r_s=-0.58, p=0.001, and r_s=-0.61, p<0.001 respectively) and RV basal diameter (r_s=0.45, p=0.02, and r_s=0.6, p=0.001 respectively) (Figure 5). Linear regression confirmed the correlation between cell surface and intracellular NHE3 expression and RV parameters, and standardized β coefficient are presented in Table 3.

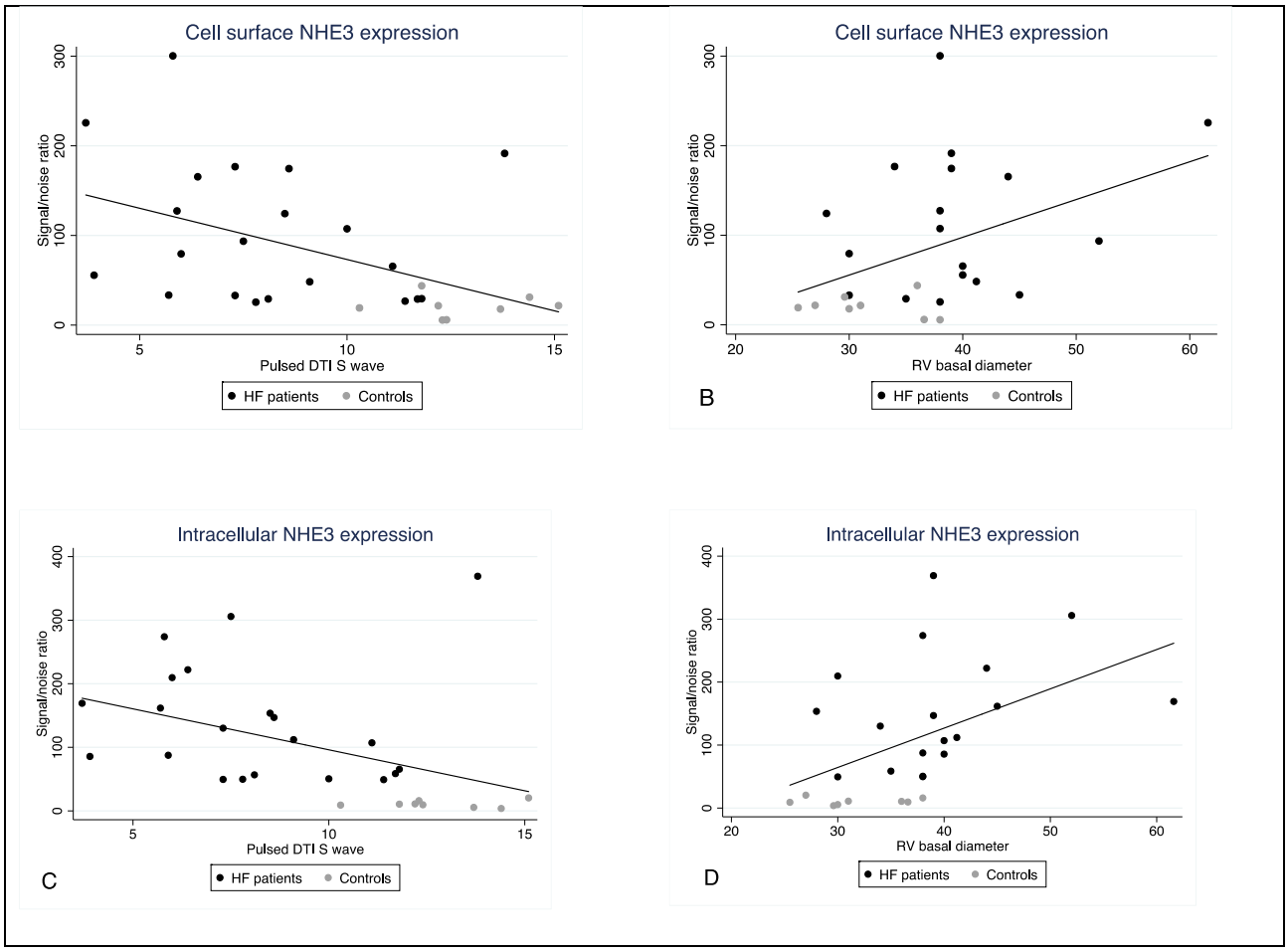

Figure 5. Correlation between cell surface and intracellular NHE3 expression, and RV parameters

Table 3: Correlation between right ventricular parameters and colonocyte NHE3 expression on linear regression analysis in heart failure patients

	TAPSE		**RV s' velocity**		**RV fractional area change**		**RV basal diameter**	
NHE3 parameters	β-coeff (95% CI)*	p-value	β-coeff (95% CI)*	p-value	β-coeff (95% CI)*	p-value	β-coeff (95% CI)*	p-value
Cell surface S/N	47 (22, 72)	0.001	37 (10, 64)	0.008	26 (-12, 64)	0.164	33 (3, 62)	0.03
Cell surface MFI	608 (335, 880)	<0.001	543 (267, 820)	<0.001	377 (-38, 792)	0.073	411 (72, 749)	<0.001
Intracellular S/N	49(13, 84)	0.01	42 (6, 77)	0.022	44 (-2, 90)	0.059	48 (12, 85)	0.009
Intracellular MFI	1616 (436, 2796)	0.009	1592 (479, 2706)	0.007	1593 (227, 2691)	0.025	1725 (537, 2913)	0.006

*Per 1-SD decrease in TAPSE, RV s' velocity, and RV fractional area change; per 1-SD increase in RV basal diameter
TAPSE = tricuspid annular plane systolic excursion; RV = right ventricular; CI = confidence interval; S/N = signal-to-noise ratio; MFI = median fluorescence intensity

DISCUSSION

Venous congestion is a hallmark of HF, and has proven to be associated with a worse prognosis including HF hospitalization and mortality. It's a major determinant of the cardiorenal syndrome, and HF-induced hepatic and gastro intestinal dysfunction. The colon plays an important role in maintaining fluid balance, by absorbing salt and water through its main sodium channel, NHE3 and could be implicated in venous congestion in HF. In the present work, we demonstrate that the expression of apical, and intracellular NHE3 protein in isolated colonocytes is increased in AHF patients, compared to hospitalized non-HF controls.

Numerous pre-clinical studies have focused on NHE3 regulation, either in the kidney or in the intestine, and has shown an acute and chronic regulation by the renin-angiotensin system, and the sympathetic system.[23,33-35] Acutely, angiotensin II increases NHE3 activity, using translational and post translational mechanisms such as trafficking from endosomal compartment, to the apical membrane, and phosphorylation. Over time, angiotensin II stimulates transcriptional activation of the NHE3 gene, resulting in increased NHE3-mRNA and total protein abundance. These transcriptional mechanisms seem to involve several signaling pathways, including p38 MAPK, ERK 1/2 and NF-κB.[36] A study in rats with HFrEF showed an increased NHE3 activity, NHE3-mRNA and protein expression in proximal renal tubule.[37] However our study is the first clinical research focusing on intestinal NHE3 expression in human HF patients. Although increased neurohormones can explain the upregulation of NHE3 expression in experimental models, it might not be enough to explain its upregulation in a HF population. Indeed, more than 50% of the HF patients in our study were treated with neurohormonal inhibitors (beta-blocker (67%), ACE or ARB (58%)). *In vitro* studies

showed that angiotensin II did not upregulate NHE3 expression when cells were pre-treated with an ARB (losartan). Another mechanism that has proven to be implicated in upregulating intestinal NHE3 expression, and could be implicated in AHF is metabolic acidosis. Intestinal ischemia, as a result of increased intestinal and systemic congestion could promote regional metabolic acidosis and upregulate intestinal NHE3, especially in HF with RVD, as RVD increases venous congestion and intestinal congestion. Indeed, in the present work, we demonstrate a strong correlation between NHE3 expression and RV parameters (size and function). Moreover, our investigative group has previously found that in patients with AHF, markers of RVD (e.g., decreased right atrial and RV function) are associated with higher levels of fatty acid binding protein (I-FABP), a marker of intestinal ischemia and gut permeability.[38] Measurements of the intracellular pH within colonocytes could help validate that hypothesis, and additional studies need to be conducted to help clarify the NHE3 upregulation mechanisms in HF with RVD.

In addition to worsening venous congestion by reabsorbing salt and water, intestinal NHE3 upregulation in AHF might have harmful effects on the gut. Thus, NHE3 is part of the cation/proton antiporter. Absorption of Na+ from the apical membrane is accompanied by extrusion of H^+ into the gut lumen, creating a luminal acidosis, that may be responsible for alteration of the gut microbiota (dysbiosis). The study of the role of microbiota in cardiovascular diseases has been increasing recently, and several studies suggest that HF patients harbor altered gut microbiota.[29,30] Furthermore, increasing evidences suggests that a permanent alteration in the microbiota composition or function can alter intestinal mobility and permeability, thus leading to an alteration of the intestinal barrier function. This intestinal permeability

may promote translocation of pathogenic microorganisms and bacterial endotoxins, such as lipopolysaccharide (LPS) into the bloodstream, promoting systemic inflammation leading to worsening myocardial dysfunction.[39,40] Supporting this hypothesis, studies have found increased serum endotoxin concentration, in patients with acute and chronic HF.[41] Our results are important because they further our understanding of the role played by the gut in HF. More studies will be necessary to highlight the relationship between NHE3 expression and gut dysbiosis. As we expand studies of colonocyte NHE3 expression in HF, it will be interesting to determine whether there are certain fecal microbiome profiles that are altered in the setting of increased NHE3 expression, and whether the resultant dysbiosis (if present) is associated with mechanisms that could worsen HF (e.g., increased inflammation).

Several limitations of our study should be noted. Even if we demonstrated that HF patients exhibit higher intestinal NHE3 expression, we didn't measure NHE3 activity. However, previous studies conducted in experimental models showed that increased NHE3 expression was paralleled by increased NHE3 activity. Moreover, as described before, an increase in NHE3 expression during HF can result from different acute and chronic mechanisms. Even though we included HF patients with ADHF, we demonstrated that both apical and intracellular NHE3 expression were higher, indicating that total NHE3 expression was higher. This suggests that upregulation of NHE3 expression was not only resulting from post-traductional mechanisms such as trafficking from endosomal compartment to the apical membrane, but that more chronic regulation mechanisms might have been involved. Measuring NHE3-mRNA, as well as studying NHE3 expression in stable HF patients could help investigate the role of acute and chronic upregulation of NHE3 in HF. Another limitation to our study is that HF patients had a relatively severe HF phenotype with a high prevalence of RVD

(Among the 24 HF patients, 14 (58%) had TAPSE<17mm, 6 (25%) had TAPSE>17mm and we couldn't assess TAPSE on 4 patients because of bad echocardiography quality). As we showed, NHE3 expression is strongly correlated to RV parameters, and we might have overestimated the importance of NHE3 expression's upregulation.

Perspectives

Intestinal NHE3 regulation is a promising target, as *in vivo* studies showed in a rat model with chronic kidney disease with hypervolemia (5/6th nephrectomy [NPX] rat model fed with a high-salt diet).[42] In these rats, initiation of tenapanor, an oral inhibitor or NHE3 activity, designed to act locally in the intestine, induced decreased urinary sodium concentration and increased fecal sodium concentration, supporting the idea that tenapanor reduces intestinal sodium uptake. Tenapanor also prevented and reversed extracellular volume expansion, decreased blood pressure and disminished left ventricular hypertrophy. A phase 1 study conducted in healthy human subjects treated with tenapanor showed an increase in fecal sodium and decrease of similar magnitude in urinary sodium, similar to the effects observed in rats, suggesting that tenapanor could provide a clinical benefit to patients with volume overload.

CONCLUSION

Colonocyte NHE3 expression is higher in ADHF patients compared to hospitalized non-HF controls, and correlates with the extent of RVD. These findings suggest that NHE3, which results in increased sodium resorption in the gut, may be a novel therapeutic target in HF.

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ANNEXES

ANNEXE 1 : Feuille d'information et consentement du patient

IRB #: STU00204053 Approved by NU IRB for use on or after 2/23/2018 through 2/22/2019.

Permission to Take Part in a Human Research Study
Do not sign this consent if today's date is later than the stated expiration date above.

Title of Research Study: *VIsceral CongesTiOn in Heart FailuRe StudY (VICTORY)*
STU00204053

Investigator: Sanjiv J. Shah, MD

Supported By: This research is supported by Northwestern University.

Financial Interest Disclosure: The following disclosure is made to give you an opportunity to decide if this relationship will affect your willingness to participate in this research study:

If your doctor is also the person responsible for this research study, please note that s/he is interested in both your clinical care and the conduct of this research study. You have the right to discuss this study with another person who is not part of the research team before deciding whether to participate in the research.

Why am I being asked to take part in this research study?

Key Information:
The first few pages of this document include a summary of this study to help you decide whether or not to participate. Detailed information is provided after the summary.

We are asking you to take part in this research study because you have been hospitalized for a heart problem

What should I know about a research study?

- Someone will explain this research study to you.
- Whether or not you take part is up to you.
- You can choose not to take part.
- You can agree to take part and later change your mind.
- Your decision will not be held against you.
- You can ask all the questions you want before you decide.

Why is this research being done?
The purpose of this research is to better understand why some patients with congestive heart failure have worsening symptoms and are hospitalized. Specifically we would like to understand how the role of the gastrointestinal system in handling fluid in the body and affecting how the blood vessels in the body work. We hope that this research will one day lead to the development of treatments that will help patients with heart failure.

The gastrointestinal tract (gut) is host to billions of microorganisms (e.g., bacteria) which we are beginning to realize play a role in health and disease. This study aims to determine how changes in the environment of the gut may play a role in fluid retention and worsening kidney disease in patients with heart failure.

How long will the research last and what will I need to do?
We expect that you will be in this research study for 30 days. The study itself will only require 2 visits (today's visit, and 1 follow-up visit approximately 30 days after you are discharged from the hospital).

Permission to Take Part in a Human Research Study
Do not sign this consent if today's date is later than the stated expiration date above.

You will be asked to provide blood samples, urine samples, and finally a stool sample. During that visit we will perform a brief ultrasound on your heart and abdomen. We will also ask to perform a simple non-invasive procedure called arterial tonometry, which records the pressure waves in your arteries.You also may be asked to complete an MRI.

More detailed information about the study procedures can be found under the section **What happens if I say "Yes, I want to be in this research"?**

Is there any way being in this study could be bad for me?

All procedures outlined in this study protocol impose minimal risk. The full-body MRI may result in shortness of breath while you will lie on your back for approximately 1 hour during the procedure. We will ask you several times before receiving the MRI if you are able to tolerate this. Claustrophobia may also be a concern to you and other participants. We will ask you if this will be an issue, and if you are not confident then you may withdraw from the MRI portion of the study at any time. There are no known risks to echocardiography or arterial tonometry. However, you may also develop mild discomfort from the echocardiography probe pressing against your chest wall and abdomen, or from the arterial tonometry wand pressing against your neck. The blood sampling requires a needle in your vein, which may cause discomfort or bruising. Finally, there could be a loss of confidentiality because of your participation in this procedure. We will take precautions to minimize the risk of loss of confidentiality.

Will being in this study help me any way?

There are no benefits to you from your taking part in this research. We cannot promise any benefits to others from your taking part in this research.

What happens if I do not want to be in this research?

Participation in research is completely voluntary. You decide whether or not to participate. If you choose to not participate, there will be no penalty to you or loss of benefit to which you are entitled.

Your alternative to participating in this research study is to not participate.

Detailed Information:
The rest of this document includes detailed information about this study (in addition to the information listed above).

Who can I talk to?

If you have questions, concerns, or complaints, or think the research has hurt you, talk to the research team at 312-695-4481, or stephen.dvorak@northwestern.edu

This research has been reviewed and approved by an Institutional Review Board (IRB). You may talk to them at (312) 503-9338 or irb@northwestern.edu if:

- Your questions, concerns, or complaints are not being answered by the research team.
- You cannot reach the research team.
- You want to talk to someone besides the research team.

Permission to Take Part in a Human Research Study
Do not sign this consent if today's date is later than the stated expiration date above.

- You have questions about your rights as a research participant.
- You want to get information or provide input about this research.

How many people will be studied?
We plan to enroll 100 patients into this research study.

What happens if I say "Yes, I want to be in this research"?
If you decide to participate in this research, we will ask you to take part in 2 visits. The first visit will take place in the hospital while you are still hospitalized. During that visit we will perform a brief ultrasound on your heart and abdomen, and ask you to provide blood samples, urine samples, and finally a stool sample. We will also ask to perform a simple non-invasive procedure called arterial tonometry, which records the pressure waves in your arteries.You also may be asked to complete an MRI. All of these procedures (except the MRI) will take place on the day of study enrollment (during your hospitalization), and will occur in your hospital room.

We will then ask if you would be willing to come in for a follow up visit after you have been discharged from the hospital. During the 30-day follow-up visit, we will repeat all of the procedures done on the day of enrollment, except that we will allow you to collect the stool sample from the privacy of your home if you would like. The following is a description of the procedures to be completed for this study:

- **Echocardiography**: An echocardiogram is an ultrasound of your heart, which involves an ultrasound probe pressing against your chest wall. We will also be taking some ultrasound pictures from your abdomen as part of the echocardiogram test.This procedure will take 15-20 minutes. .
- **Blood sampling:** We will obtain blood samples from one of your veins by a trained phlebotomist. We will take approximately 2 tablespoons of blood.
- **Arterial tonometry**: This is a simple non-invasive test in which we will examine the stiffness of your blood vessels. It involves placing blood pressure cuffs on your arm and leg and also a small wand (the size of a pencil) on your neck artery where we will apply gentle pressure. This procedure takes approximately 10 minutes. There are no risks involved with this procedure.
- **Stool sample**: We will provide you with a paper "hat" which can be suspended in the toilet to provide a stool sample. We will ask you to deposit a portion of the total specimen into a plastic container provided by us (we will do this for you if you are unable to do it)
- **Urine sample**: We will ask you to provide a urine sample in a plastic cup.
- **MRI**: We may ask you to undergo a non-contrast MRI scan of your body to allow us to better see how the blood flows in the major arteries and veins of your abdomen. This MRI procedure will take approximately 1 hour.

Visit #2 will include the same procedures as the ones listed above except we will offer you the stool sample kit to be done at home if that is more comfortable for you.

What happens if I say "Yes", but I change my mind later?

You can leave the research at any time; it will not be held against you.

Permission to Take Part in a Human Research Study
Do not sign this consent if today's date is later than the stated expiration date above.

If you decide to leave the research, contact the investigator so that the investigator can retrieve any equipment that may had been left with you such as the pulse wave analysis watch. Otherwise there is no risk for leaving the study at any time.

Choosing not to be in this study or to stop being in this study will not result in any penalty to you or loss of benefit to which you are entitled. Specifically, your choice not to be in this study will not negatively affect your right to any present or future medical treatment.

If you stop being in the research, already collected data may not be removed from the study database. You will be asked whether the investigator can collect data from your routine medical care.

If you agree, this data will be handled the same as research data.

Detailed Risks: Is there any way being in this study could be bad for me?

All procedures outlined in this study protocol impose minimal risk. The full-body MRI may result in shortness of breath while you will lie on your back for approximately 1 hour during the procedure. We will ask you several times before receiving the MRI if you are able to tolerate this. Claustrophobia may also be a concern to you and other participants. We will ask you if this will be an issue, and if you are not confident then you may withdraw from the MRI portion of the study at any time. There are no known risks to echocardiography or arterial tonometry. However, you may also develop mild discomfort from the echocardiography probe pressing against your chest wall and abdomen, or from the arterial tonometry wand pressing against your neck. The blood sampling requires a needle in your vein, which may cause discomfort or bruising. Finally, there could be a loss of confidentiality because of your participation in this procedure. We will take precautions to minimize the risk of loss of confidentiality.

This study involves the use of your identifiable, personal information and there is a chance that a loss of confidentiality could occur. The researchers have procedures in place to lessen the possibility of this happening. See the section below titled: "What happens to the information collected for the research?".

Will it cost me anything to participate in this research study?

Taking part in this research study will not lead to any costs to you.

What happens to the information collected for the research?

Efforts will be made to limit the use and disclosure of your personal information, including research study and medical records, to people who have a need to review this information. We cannot promise complete secrecy. Organizations that may inspect and copy your information include the IRB and other representatives of this institution.

Data and specimens saved from this study will be stored in the clinical research lab of Dr. Sanjiv Shah, in a locked office and freezer. Members of the investigation team will have access to those data. The specimens will be retained for 10 years after the study for the purposes of future research. These specimens will be de-identified so that your personal health information will not be associated with these specimens.

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HIPAA Authorization

We are committed to respect your privacy and to keep your personal information confidential. When choosing to take part in this study, you are giving us the permission to use your personal health information that includes health information in your medical records and information that can identify you. For example, personal health information may include your name, address, phone number or social security number. Your health information we may collect and use for this research includes:

- All information in a medical record
- Results of physical examinations
- Medical history
- Lab tests, or certain health information indicating or relating to a particular condition as well diaries and questionnaires
- Billing information

The following clinical providers may give the researchers information about you: all current and previous health care providers, including but not limited to the Shirley Ryan AbilityLab (SRALAB), Northwestern Medical Group (NMG), Northwestern Memorial Hospital (NMH), and Northwestern Lake Forest Hospital (NLFH).

Once we have the health information listed above, we may share some of this information with the following offices or entities outside of Northwestern University and its clinical partners (or affiliates): the Northwestern University Institutional Review Board Office and Office for Research Integrity; the US Office of Research Integrity; the US Office for Human Research Protections; the US Food and Drug Administration.

Any research information shared with outside entities will not contain your name, address, telephone or social security number or any other personal identifier unless disclosure of the identifier is necessary for review by such parties or is required by law or University policy [except that such information may be viewed by the Study sponsor and its partners or contractors at the Principal Investigator's office].

- Authorized members of the Northwestern University workforce, who may need to see your information, such as administrative staff members from the Office for Research, Office for Research Integrity and members of the Institutional Review Board.
- Clinical affiliates, including but not limited to the Shirley Ryan AbilityLab (SRALAB), Northwestern Medical Group (NMG), Northwestern Memorial Hospital (NMH), Northwestern Lake Forest Hospital (NLFH), and the Ann & Robert H. Lurie Children's Hospital of Chicago (Lurie Children's). Your participation in this clinical trial may be tracked in an electronic database and may be seen by investigators running other trials that you are enrolled in and by your healthcare providers.
- Clinical affiliates, including but not limited to Northwestern Medical Group (NMG), Northwestern Memorial Hospital (NMH), and Northwestern Lake Forest Hospital (NLFH), for purposes including, but not limited to, the affiliate's provision of care to you and/or the affiliate's scheduling of appointments and/or billing activities.

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- Other University research centers and University contractors who are also working on the study,
- Study monitors and auditors who make sure that the study is being done properly,
- Government agencies and public health authorities, such as the Food and Drug Administration (FDA) and the Department of Health and Human Services (DHHS).

Those persons who get your health information may not be required by Federal privacy laws (such as the Privacy Rule) to protect it. Some of those persons may be able to share your information with others without your separate permission.

However, Illinois law does not allow the re-release of HIV/AIDS, genetic testing, mental health and developmental disabilities information by the recievers of the information except in precise situations allowed by law.

Also, Federal Confidentiality Rules, 42 CFR Part 2, prohibit making any further disclosure of substance use disorder information unless further disclosure of this information is expressly permitted by written consent of the person to whom it pertains or as otherwise permitted by 42 CFR Part 2.

The results of this study may also be used for teaching, publications, or for presentation at scientific meetings.

Unless you revoke your consent, it will expire in 60 days from initial signing.

Although you may revoke consent to participation in this research at any time and in any format, you must revoke authorization for use or disclosure of your health information in writing. To revoke your authorization, write to:

PI's Name: Sanjiv Shah, MD
Institution: Northwestern University
Department: Cardiology
Address: NMH/Arkes Family Pavilion Suite 600
676 N Saint Clair
Chicago IL 60611

You do not have to authorize the use or disclosure of your health information; however, you will not be allowed to take part in this research study. If you do not authorize the use or disclosure of your health information, it will not affect your treatment by health care providers, or the payment or enrollment in any health plans, or affect your eligibility for benefits.

However, Illinois law does not allow the re-release of HIV/AIDS, genetic testing, mental health and developmental disabilities information by the recievers of the information except in precise situations allowed by law.

Also, Federal Confidentiality Rules, 42 CFR Part 2, prohibit making any further disclosure of substance use disorder information unless further disclosure of this information is expressly permitted by written consent of the person to whom it pertains or as otherwise permitted by 42 CFR Part 2.

Permission to Take Part in a Human Research Study
Do not sign this consent if today's date is later than the stated expiration date above.

A copy of this signed consent document, information about this study, and the results of any test or procedure done may be included in your medical records and may be seen by your insurance company.

Optional Elements:
The following research activities are optional, meaning that you do not have to agree to them in order to participate in the research study. Please indicate your willingness to participate in these optional activities by placing your initials next to each activity.

I agree	I disagree	
_____	_____	The researcher may contact me in the future to see whether I am interested in participating in other research studies by the Principal Investigator of this study.
_____	_____	The researcher may retain any leftover blood or tissue samples taken during the study. These samples may be used for other research not related to this study. These samples will be retained in non-identifiable form, meaning that there will be no information associated with the blood or samples that will allow anyone to readily ascertain my identity.

Your signature documents your permission to take part in this research. You will be provided a copy of this signed document.

_____ _____
Signature of Participant Date

_____
Printed Name of Participant

_____ _____
Signature of Person Obtaining Consent Date

_____
Printed Name of Person Obtaining Consent

ANNEXE 2 : Protocole d'isolation des colonocytes exfoliés (SCSR kit isolation)

A. Preparation

1. Store the stool samples collected in 15 ml of transport medium in **pre-weighed** 30 ml screw capped fecal collection tubes. (We recommend the samples be processed within 7 days of collection. If the samples cannot be processed immediately, store them at 4ºC. Samples can be transported at room temperature a maximum of 7 days. The 15 ml volume is designed to preserve a 0.5 - 1.0 g sample.
2. Pre-warm the SCSR dispersing medium (SCSR-T) and cushion solution (SCSR-C) to room temperature.
3. Record the weight of the tubes containing the fecal samples.
4. Calculate the weight of the fecal sample by subtracting the weight 'before collection' from the weight 'after collection' (*If the total weight of the fecal sample is more than 1 g, add 10 ml of transport medium (SCSR-T) to the tube containing sample. This will speed up filtration in Step 10.*

B. Strain and Filter Sample

6. Make sure the sample tubes are tightly closed and do not leak, then vortex to suspend the sample.
7. Working in the hood, transfer the sample into a 330 µm Stomacher strainer bag
8. Press the bag to help the sample through the strainer into the outer bag
9. Draw out filtered sample using a plastic 25 ml pipette from the plastic bag into a 50 ml labeled tube
10. Filter the sample through 40 µm filter into pre-labeled 50 ml centrifuge tube. If this process is slow, use pipette to unclog the filter by repeatedly aspirating and dispensing the sample into the filter. It may be helpful to tip the filter at a 30deg angle
11. Calculate the volume of the sample equivalent to 0.5 g of the original fecal sample.
12. Remove the filtered sample equivalent to 0.5 g of fecal sample into another 50 ml tube using a pipette.

C. Underlay Cushion and Centrifuge

13. Adjust the total volume to 25 ml with transport medium (SCSR-T) and mix by vortexing.
14. Carefully underlay the sample with 10 ml cushion solution (SCSR-C), pre-warmed to room temperature using a 10 ml pipette
15. Spin the samples at 200xg for 10 minutes at room temperature

D. Extract Fractions

16. Remove supernatant (if desired, save an aliquot for studying extracellular microbes), and discard into bleach-containing waste bottle.

(*To maximize cell recovery, leave approximately 5 ml of supernatant above the interphase.*)

17. Carefully remove the interphase into a pre-labeled sterile 50 ml tube. (*To maximize cell recovery, it is permissible to include 5 ml of the supernatant from above the interphase. It is permissible to include a small amount of the cushion with the interphase.)* \
18. Leave the cushion in the tube containing the pellet. The cushion will be washed away in subsequent steps.

E. Wash Cell Fractions

19. Wash cell fractions (ie interphase and pellet fractions) by adding cold PBS pH 7.2 to each of the tubes, adjusting the total volume to 40 ml. Re-suspend by either inverting the tubes several times or gently vortexing. Spin the cells at 900xg at 4ºC for 10min. Carefully aspirate the supernatant and dispense into a container containing bleach.
20. Repeat wash twice (Step 19), using 15 ml aliquots of PBS.
21. Re-suspend the pellet in 1 ml cold PBS per 0.5 g of original fecal sample and place the cell suspension on ice.

F. Count Cells and Aliquot

22. To disaggregate clumped cells, pass cell suspension through a 20 G needle 10 times while the tubes are on ice.
23. Mix the cells by tapping the tube gently to make the cells suspension homogenous.
24. Count the cells in a Coulter counter using 2-5 µm and 5-8 µm ranges.
25. Prepare aliquots according to desired cell counts. *(We recommend using the 5-8 µm cell counts as a basis)*

G. Freeze

26. Spin the tubes containing cells in a refrigerated centrifuge at 900xg for 5 min.
27. Remove supernatant and re-suspend cells in 100 µl serum-free cell freezing medium. Make sure to suspend the pellet by gently mixing after adding the freezing medium.
28. Store the aliquots of colonocytes at -80ºC.

ANNEXE 3 : Protocole de préparation cellulaire pour la cytométrie de flux

Protocol: Antibody Labeling with Indirect Fluorochrome Conjugation

1. Thaw frozen cells and wash twice with PBS+2%BSA. (Centrifuge at 1500 rpm for 10 minutes, 4 ºC, remove the supernatant using pasteur pipette). Resuspend cells at a concentration of approx. 1 million cells per 100uls in PBS+2%BSA.
2. Add 100uls of single cell suspension in each tube and process of flow staining. Include appropriate controls for the experiment (negative unstained control, primary antibody alone and secondary antibody alone controls).
3. Add 1 µl of primary anti-NHE3 antibody to the respective sample tubes.
4. Incubate the cells at room temperature for 30 minutes or 4 ºC for 60 minutes.
5. Add 3 ml of PBS+2%BSA wash buffer, suspend the cells gently by tapping the tube.
6. Centrifuge at 1500 rpm for 10 minutes at 4 ºC and remove the supernatant using pasteur pipette.
7. Repeat step 5 and 6 once more.
8. Suspend the cells gently in 100 µl of 1% BSA in PBS by tapping the tube.
9. Add 5 µl of secondary antibody with conjugate (1:1500 diluted).
10. Incubate the cells at room temperature for 30 minutes or 4 ºC for 60 minutes.
11. Add 3ml PBS+2%BSA, suspend them gently by tapping tube.
12. Centrifuge at 1500 rpm for 10 minutes at 4 ºC and remove the supernatant using pasteur pipette.
13. Repeat step 11 and 12 once more.
14. Suspend the cells in 0.5 ml of 1%BSA in PBS.
15. Analyze using flow cytometer.

Protocol: For Intracellular preparation followed by Antibody Labeling with Indirect Fluorochrome Conjugation

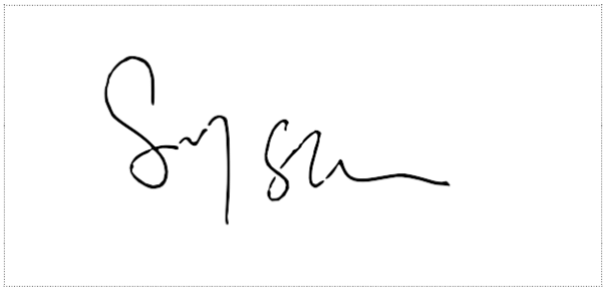
1. Thaw frozen cells and wash twice with PBS. Centrifuge at 1500 rpm for 10 minutes, 4 ºC, remove the supernatant using Pasteur pipette. Resuspend cells at a concentration of approx. 1 million cells per 100uls.
2. Add 100uls of single cell suspension in each tube and process of cell fixation and permeabilization as below. Include appropriate controls for the experiment (negative unstained control, primary antibody alone and secondary antibody alone controls).
3. Add 65uls of 10% Formaldehyde to each tube, vortex gently and incubate at room temperature for 10 mins.
4. Centrifuge at 2500 rpm for 10 minutes, 4 ºC, remove the supernatant using pasteur pipette.
5. Add 1 ml PBS, suspend them gently by tapping tube.
6. Centrifuge at 2500 rpm for 10 minutes, 4 ºC, remove the supernatant using pasteur pipette.
7. Incubate the cells with 2 ml of 1% BSA, 0.1% Triton-X 100 solution in PBS for 10 minutes
8. Add 2 ml of PBS+2%BSA wash buffer, suspend the cells gently by tapping the tube.
9. Centrifuge at 2500 rpm for 10 minutes at 4 ºC and remove the supernatant using pasteur pipette.
10. Repeat wash steps 8-9 once.
11. Resuspend cells in 100uls of PBS+2%BSA and proceed to staining protocol.

Vu, le Président du Jury,

(tampon et signature)

Professeur Jean-Noël TROCHU

Vu, le Directeur de Thèse,
(tampon et signature)



Professeur Sanjiv SHAH

Vu, le Doyen de la Faculté,

Professeur Pascale JOLLIET

ABSTRACT

Background Venous congestion has been associated with increased morbidity and mortality in heart failure (HF). Sodium/hydrogen exchanger 3 (NHE3), the main sodium channel in the colon, helps to maintain fluid balance but may be maladaptative mechanism underlying fluid overload in HF. In experimental models, NHE3 is upregulated by factors known to be increased in HF: acidosis, angiotensin II and catecholamines. However, the role of NHE3 in human HF has not yet been elucidated.

Objectives We hypothesized that NHE3 protein expression is upregulated in acute HF patients (AHF), compared to hospitalized controls with cardiovascular comorbidities but without HF. We also hypothesized that within acute HF patients, the degree of right ventricular dysfunction (RVD) correlates with the extent of NHE3 expression.

Methods Twenty-four patients admitted for ADHF, and 10 controls (hospitalized patients without HF) were included. Exfoliated colonocytes were isolated from stool samples, collected during hospitalization. Cell surface, and intracellular NHE3 expression was measured using flow cytometry, and expressed as signal to noise ratio (S/N). Echocardiography was performed in all study patients to evaluate cardiac structure/function.

Results Cell surface and intracellular NHE3 expression was significantly higher in HF patients compared to controls (median [IQR] S/N = 79.4 [29.8, 165.4] vs 21.3 [17.8, 31.1], $p<0.001$ and 112.0 [58.6, 169.3] vs 10.1 [7.7, 13.6], $p<0.001$ respectively), even after adjusting for age, hypertension, atrial fibrillation, coronary artery disease, medications (angiotensin converting enzyme, angiotensin receptor blockers, beta-blockers, and corticosteroids) and estimated glomerular filtration rate (GFR) ($p<0.001$). Moreover, cell surface and intracellular NHE3 expression was highly correlated with RVD, and RV enlargement: TAPSE ($r=-0.60$, $p<0.001$, and $r=-0.56$, $p=0.002$ respectively); basal RV free wall tissue Doppler systolic (s') velocity ($r=-0.58$, $p=0.001$, and $r=-0.61$, $p<0.001$ respectively); and RV basal diameter ($r=0.45$, $p=0.02$, and $r=0.6$, $p=0.001$ respectively).

Conclusion Colonocyte NHE3 expression is higher in ADHF patients compared to hospitalized non-HF controls, and correlates with the extent of RVD. These findings suggest that NHE3, which results in increased sodium resorption in the gut, may be a novel therapeutic target in HF, particularly in patients with right-sided HF.

Key words

Acute heart failure, right ventricular dysfunction, venous congestion, splanchnic congestion, sodium/hydrogen exchanger 3

NOM : MARTEAU PRENOM : Lara

Titre de Thèse : EXPRESSION DE L'ÉCHANGEUR NHE3 AU NIVEAU DU COLON DANS L'INSUFFISANCE CARDIAQUE AIGUE

RESUME

Introduction : L'échangeur sodium/hydrogène 3 (NHE3), principal échangeur sodique du colon, participe au maintien de la balance hydrosodée. Il pourrait par des mécanismes maladaptatifs, être surexprimé dans l'insuffisance cardiaque aigue (ICA), aggravant la congestion veineuse. L'objectif de cette étude est de montrer une surexpression de NHE3 colique chez des patients hospitalisés en ICA comparés à des contrôles, et de montrer que cette surexpression est corrélée à la fonction ventriculaire droite.

Matériel et méthodes : Il s'agit d'une étude prospective, contrôlée, monocentrique. Le critère de jugement principal était l'expression de l'échangeur NHE3 membranaire et intracellulaire, mesurée en cytométrie de flux (ratio signal/bruit [S/N]) sur des colonocytes exfoliés isolés sur un prélèvement de selles.

Résultats : 34 patients hospitalisés en ICA, et 10 contrôles ont été inclus. L'expression de NHE3 membranaire et intracellulaire était supérieure dans le groupe de patients ICA comparés aux contrôles (ratio S/N médian = 79.4 [29.8, 165.4] vs 21.3 [17.8, 31.1], p<0.001 et 112.0 [58.6, 169.3] vs 10.1 [7.7, 13.6], p<0.001 respectivement). L'augmentation de l'expression de NHE3 était corrélée à l'importance de dysfonction ventriculaire droite (TAPSE (r=-0.60, p<0.001, et r=-0.56, p=0.002 respectivement).

Conclusion : L'expression de NHE3 colique est augmentée chez des patients en ICA, et est corrélée à l'importance de la dysfonction ventriculaire droite.

MOTS-CLES

Insuffisance cardiaque aigue, dysfonction ventriculaire droite, congestion veineuse, congestion splanchnique, échangeur sodium/hydrogène 3 colique