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Non Invasive Evaluation of Cardiac Hemodynamics in End Stage Renal Disease (ESRD).

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/149864> since

Published version:

DOI:10.1007/s40292-014-0045-4

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UNIVERSITÀ DEGLI STUDI DI TORINO

This is a non-final version of an article published in final form in

Milan A, Avenatti E, Della Valle E, Fabbri A, Ravera A, Pozzato M, Ferrari G, Quarello F, Aprà F, Veglio F (2014) **Non Invasive Evaluation of Cardiac Hemodynamics in End Stage Renal Disease (ESRD).**, HIGH BLOOD PRESSURE & CARDIOVASCULAR PREVENTION
(ISSN:1120-9879)

The definitive version is available at:

DOI 10.1007/s40292-014-0045-4

Non invasive evaluation of cardiac hemodynamics in end stage renal disease (ESRD)

Short title: Fluid overload assessment

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The present manuscript has been seen and approved by all authors and it is not under consideration for publication elsewhere in a similar form, in any language, except in abstract form.

Conflict of interest: None

Abstract

Objective: Volume overload is typical of haemodialysis patients; correct volume status evaluation is crucial in achieving blood pressure homeostasis, hypertension management and good treatment planning. This study evaluates the effect of acute volume depletion on ultrasonographic parameters and suggests two of them as able to predict patients volume overload.

Patients and Intervention : 27 patients with end stage renal disease treated with haemodialysis underwent a complete echocardiographic exam before, after 90 minutes and at the end of the dialysis.

Main outcome and Results: Blood pressure levels significantly drop during the first 90 minutes of dialysis (139 ± 20 vs 126 ± 18 ; $p<0.0001$), reaching a steady state with significantly lower values compared to baseline (130 ± 28 ; $p = 0.02$). LV and left atrial volume significantly decreased (baseline vs end dialysis 98 ± 32 vs 82 ± 31 $p = 0.003$ and 28 ± 10 vs. 21 ± 9 cc/m² $p<0.001$). A significant reduction of systolic function (EF $61.6\%\pm 9$ vs $58.7\%\pm 9$ $p=0.04$), of diastolic flow velocities (E/A 1.13 ± 0.37 vs 0.87 ± 0.38 $p<0.001$) and mitral annulus TDI tissue velocity (i.e. E'lat 10.6 ± 3 vs. 9.4 ± 3 cm/s; $p 0.0001$) were observed. Stroke work (SW) and LV end-diastolic diameter (LVEDd) indexed to height 2.7 (LVEDdi) were able to predict volume overload: cut off values of respectively 13.5 mm/m^{2.7} for LVEDdi and 173 cJ for SW were able to predict with a specificity of 100% the presence of a volumic overload of at least 4%.

Conclusions: Blood pressure, cardiac morphology and function are significantly modified by acute volume depletion and such variations are strictly interrelated. SW and LVEDd/height^{2.7} may identify ESRD patients carrying an higher volume load.

Keywords: Dry weight, Echocardiography, Haemodialysis, Volume overload

Introduction

The evaluation of volemic status plays a pivotal role in patients affected by end stage renal disease (ESRD) in need of haemodialysis (HD) treatment. Chronic volume overload in ESRD has since time been recognized as the main responsible of hypertension development, with its detrimental role in increasing incidence of cardiovascular disease [1,2]. Moreover, HD-induced volume depletion is related to intradialytic hypotension [3], an independent risk factor for fatal events in ESRD population [4]. Definition of real volume overload before HD session may therefore represent a key point for minimizing complications and optimize treatment. Volume overload and target dehydration weight are still largely estimated empirically [5], even if different methods have been proposed, from bioimpedance to temperature monitoring and echocardiography evaluations [6]. The latter represent an appealing option, as a relatively cheap, completely harmless method able to describe not only volume status [7-10] but to picture the spectrum of ESRD cardiomyopathy as well [11-12].

Aim of the present study has been double: 1) to describe the variation of different echocardiographic parameters in the physiopathological model offered by hemodialysis – i.e. acute volume depletion - in order to confirm their dependence on preload status and its modification and 2) to identify among them, the ones able to describe and quantify volume overload in ESRD patients

Participants and Methods:

Patients: 27 patients were selected, among the population of 117 subjects undergoing thrice weekly haemodialysis (HD) for end stage renal disease in the Nephrological Department of S Giovanni Bosco Hospital in Turin. Exclusion criteria were: age > 75 or < 18 years, BMI > 40 kg/m², atrial fibrillation, more than mild valve regurgitation, any valve stenosis, impaired systolic function, hypertrophic or dilatative cardiomyopathy, hemodynamic instability . The study was approved by our Institutional Review Committee and all subjects provided their written informed consent (protocol number 08/02/11)

Study protocol: All eligible patients underwent echocardiographic evaluation immediately before HD (baseline), after 1,5 hours and at the end of the HD. Clinical parameters - systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), weight - were recorded just before every study session. Fifteen of the study subjects had the complete protocol repeated in two different HD days, and 4 patients were evaluated thrice in different treatment days with a total of 50 echocardiographic sessions available for data analysis (figure 1).

Data were firstly analysed in order to confirm variability of clinical and echocardiographic parameters in a setting of acute preload variation; and then used to identify echocardiographic parameters able to describe and quantify volume overload in HD patients. Volume overload was firstly defined as difference between

actual weight at the beginning of HD session and at the end of treatment (absolute overload). Patients were divided in three groups accordingly to tertiles of dehydration volume (mild <2.3 kg, moderate, severe >2.9 kg) and correlations between overload severity and clinical and echocardiographic variables were tested.

The same analysis was repeated indexing the absolute dehydrated volume to baseline weight, (i.e. percentage dehydration), in order to avoid the bias linked to a different height and BSA: the study population was divided in two subgroups (percentage of dehydration < or > 4%).

Haemodialysis: All treatments used a Fresenius 5008 machine (Bad Homburg, Germany) equipped with biofeedback system; dialysis parameters such as haematocrit (Hct) and dehydration velocity were recorded immediately before echocardiographic evaluation.

Echocardiography: A 2D echocardiogram was performed at rest with commercially available ultrasound systems -Esaote MyLab 45 - equipped with a 2.5-4 Mhz probe and Tissue Doppler Imaging (TDI) software. All the exams were performed by the same experienced operator (ED).

All studies comprehended parasternal long- and short-axis views and apical 4-, 5-, 2-, and 3-chamber long-axis views according to standard methods. End-diastolic and end-systolic left ventricular internal diameters (LVEDd, LVEDs), end-diastolic interventricular septum and infero-lateral wall-thickness (ILWd) were calculated from five consecutive cardiac cycles according to the current guidelines [11]. Left ventricular (LV) mass was calculated according to the Devereux formula [12], and normalized for body surface area (LVMI). LV hypertrophy was defined for values of LV mass index $\geq 110 \text{ g/m}^2$ in women, and $\geq 125 \text{ g/m}^2$ in men [13]. Relative wall-thickness (RWT) was calculated as $(2 \times \text{ILWd})/\text{LVEDd}$ and patterns of LV hypertrophy were defined according to ESH/ESC guidelines [15].

Left Atrial (LA) diameter was measured following current guidelines [13]. LA volume (LAV) was assessed by the biplane area-length method; values were normalized for body surface area.

Inferior vena cava dimensions and collapsibility index were recorded from subcostal view.

Tissue Doppler Imaging (TDI) was recorded following current guidelines [13]; measurements included systolic myocardial velocity (S'), and early and late diastolic (E' and A' respectively) myocardial velocity at the mitral annulus. All reported measurements are the averages derived from five consecutive cardiac cycles [16]. Diastolic function was assessed following current guidelines [16].

Stroke work (SW) was defined as

$$\text{SW} = 0.014 \times \text{stroke volume} \times \text{SBP},$$

being stroke volume the velocity time integral (VTI) measured at left ventricular outflow tract (LVOT):

$$\text{Stroke Volume} = 3.14 \times (\text{LVOT}/2)^2 \times \text{VTI}_{\text{LVOT}}.$$

Statistical Analysis: Statistical analysis was conducted using SAS V8 software (SAS Institute Inc. – Cary, NC, USA). The parametric distribution of the variables was analysed using the Kolmogorov Smirnov test and residual analysis. Data are expressed as mean $\pm$ Standard Deviation (SD) or as median and

interquartile difference if appropriate. Differences between means were examined using a *t* test or ANOVA for normally distributed variables. Kruskal Wallis, or non parametric ANOVA, was used for non normally distributed variables.

Statistical significance was assumed if the null hypothesis could be rejected at $p < 0.05$.

Results

Clinical, dialytic and echocardiographic features of the study population at baseline are reported in table I and II. Mean age of the study population was 55.7 ± 12.2 years; 23% of patients were diabetics and 73.9 % hypertensives. Mean ultrafiltration rate during HD sessions was 2.67 ± 0.88 liters .

Left ventricular hypertrophy was present in 42% of the study population, with different patterns (27.7% concentric, 14.3 % eccentric); left ventricle showed altered morphology, with dilated systolic (21%) or diastolic (13% of the population) volume values, but normal systolic function ($EF\ 61.6 \pm 9.1\ %$). Considering diastolic function variables, TDI values suggested an increased LV filling pressure in 37% of subjects and a left atrial dilatation in 39% of the study population.

When analyzing clinical and echocardiographic data variations during hemodialysis – table III-, dehydration implied a significant reduction of SBP ($p = 0.02$), mean blood pressure (MBP) and pulse pressure (PP) ($p = 0.01$). No relevant change occurred among the intermediate evaluation and the one performed at the end of HD.

Most echocardiographic parameters significantly changed during HD. Left ventricular mass decreased significantly, both considering absolute and indexed values. A significant reduction of LV diastolic volume occurred, without relevant variation of systolic values; a slight decrease of EF was detectable, without alteration of TDI parameters (S'). Moreover, an important reduction of the calculated SW was seen (from 127.1 ± 42.9 cJ to 93.9 ± 29.1 cJ, $p = 0.004$). All mitral Doppler parameters describing diastolic function decreased significantly (E, A, E/A ratio) in the three evaluations. A significant reduction of LA volume was detectable as well, together with reduction of TDI early diastolic filling velocity (E'), both septal and lateral, and indexes of left ventricular filling pressures (E/E').

Relation between echocardiographic parameters variation and UF amount were analysed, grouping patients at first on the basis of estimated overload and of percentage overload subsequently.

Considering estimated overload, the only clinical variable significantly associated to overload severity was age ($r_{0.5}$ $p = 0.0002$) as patients with milder overload were significantly younger - data not shown. Among echo parameters, TDI early velocities (septal $E' = 0.44$, $p = 0.03$; lateral $E' = 0.47$, $p = 0.02$; tricuspidal $E' = 0.43$, $p = 0.02$) were significantly associated to overload severity, while no correlation was detectable for E'/A' values or LV filling pressure (E/E' i.e. $r = 0.2$; $p = 0.3$). A-wave at mitral PWD was directly related to the overload degree, and consequently E/A ratio showed an inverse correlation. A direct correlation was found for LA

volume as well but inferior vena cava diameters and collapsibility index did not vary accordingly to overload severity.

Considering percentage overload (<4% or >4%) – table IV – again the only clinical variable significantly associated to overload severity was age (p 0.02) as patients with milder overload were significantly younger; no significant differences were detected among the two subgroups regarding pressure values.

Analysing echocardiographic features end diastolic LV diameter, both absolute (p = 0.01) and indexed values (p= 0.03), indexed LV mass (p=0.001) and stroke work (p=0.008) were significantly higher in the subgroup of patients showing a percentage overload >4%. No relevant difference was detectable for Doppler or Tissue Doppler parameters.

The analysed parameters were tested for association with overload severity, and end diastolic diameter indexed for height 2.7(LVEDdi) and stroke work (SW) resulted to have a good association with overload degree (r 0.41, p 0.003 for LVEDdi and r 0.44, p 0.008 for SW). Correlations between overload degree and other echocardiographic measure, such as LA supero-inferior diameter or aortic VTI, were weaker.

In a regression analysis stroke work and end-diastolic diameter resulted to be key predictors of overload severity, being able to estimate up to 30% of percentage overload variability (figure 2). A subsequent evaluation through ROC curves using as predictors the two variables - LVEDdi (AUC 70%) and SW (AUC 69%) – led to the identification of cut off values - respectively 13.5 mm/m^{2.7} for LVEDdi and 173 cJ for SW - able to predict with a specificity of 100% the presence of a volemic overload of at least 4%.

DISCUSSION

The correct evaluation of volemic status in ESRD patients is altogether a challenge and a clinical need. A non-invasive, widely applicable method for overload estimation in such clinical setting may improve patients management and survival.

Our study used an echocardiographic approach as dependence of many echocardiographic parameters from preload has been extensively debated in literature: our data confirm such high dependence. This relationship is found to be true even for the ones firstly considered to be less affected by volume status, such as tissue Doppler values. Moreover, our data suggest that among echocardiographic parameters, Stroke Work (SW) and end-diastolic diameter indexed for height 2.7(LVEDdi) are able to predict the rate of overload in the single patient.

Dehydration during HD led to reduction of all main clinical parameters (SBP, MBP, PP), in agreement with the hypothesis of the dependence of pressure values on hypervolemia in such subset [2].

In our study in particular SBP and PP at the end of HD were significantly reduced when compared to baseline data but not when comparison was made with intermediate evaluation. A different dehydration rate used during HD session, which was higher in the first hour – in contrast with the schedule of a constant UF rate of 55 cc/h used by Hung et al. [17], that demonstrated as well a significant reduction at the end of HD - and the presence of a “refilling” from interstitial space into venous system, may explain this finding. The

decrease of SBP was greater than decrease of DBP, leading to a reduced PP, a potentially interesting result in terms of cardiovascular risk profile modification.

Our data confirm that acute changes in preload conditions lead to specific significant alteration of echocardiographic parameters. Left ventricular morphology itself is heavily affected by preload condition; in particular, HD significantly reduced LV hypertrophy prevalence in our population (42% vs 24%) as well as in other studied groups [2]. This change, as recently clearly pointed out [18], is mainly due to a reduction in end-diastolic diameter, as no difference in infero-lateral wall thickness was revealed in our population. Parameters describing LV systolic function can reflect such a modification as well: a greater reduction of end-diastolic volume compared to systolic one was observed, explaining the observed EF reduction, as expected accordingly to Frank Starling law, without impairment of TDI values describing systolic function.

Atrial dimensions were affected by preload condition, accordingly to previously published studies. Parameters describing diastolic function have all been previously evaluated in HD patients. Our data are confirmatory in detecting preload sensitiveness of not only transmitral flow Doppler [7,19] but of main tissue Doppler parameters as well [9,20]. Inferior vena cava diameters as expected were significantly modified by dehydration, accordingly to previous studies [17,20,21]. In our population, IVC diameters were significantly reduced after the first one and a half hour of HD compared to baseline, while such a difference was no more present at the end of the treatment; once again, this finding may be due to the faster UF rate used in the first HD hour.

In the second part of the study the assessed preload influence on echocardiographic parameters has been used to find possible non-invasive predictors of overload severity. Standard evaluation of overload is made as difference between actual and ideal weight (“dry weight”); determination of the latter is still mostly based on clinical evaluation only [22], a method far too empiric that results in a difficult esteem of real overload severity and consequent potential harm during a too strict HD session, exposing patients to risks of intradialytic hypotension, i.e. increased mortality [23]. Moreover, use of blood pressure value measured at the time of HD session as marker of chronic volume overload severity has been questioned, as it may be heavily impaired by imprecision, and consequently represent only a very poor marker of volume overload [24].

To the best of our knowledge our work is the first in literature indicating a significant association between percentage volumic overload and two echocardiographic parameters, the end-diastolic diameter indexed for height 2.7 (LVEDdi) and stroke work (SW). The latter has been evaluated in healthy subjects undergoing acute volume load, and showed a significant increase, confirming its dependence on preload condition [25] but no data are reported regarding the reverse correlation in the specific subset of HD patients. In the multi-variate analysis we performed these two parameters were able to predict up to 30% of percentage overload variability. Sensitivity and specificity of the cut offs may be hampered by the small sample of the study, and

will need further evaluation in bigger population. However, our data demonstrated the possibility to identify the presence of a volemic overload of at least 4% using the two combined parameters with a cut-offs of 13,5 mm/m^{2.7} for LVEDdi and 173 cJ for SW. The suggested cut-offs have been chosen to give the maximum specificity, in order to grant the greater clinical usefulness.

CONCLUSION

Our results suggest that among ESRD patients undergoing HD, the combination of older age and higher LVEDd and SW on basal echocardiographic evaluation irrespectively of blood pressure values can identify the subgroup carrying an higher volume load, that may benefit from a more intensive HD program, against others that may need a softer ultrafiltration rate schedule, avoiding excessive weight loss and potentially limiting intradialytic hypotension

These three parameters may therefore guide monitoring and therapy of HD patients, in order to tailor therapy and optimize results in terms of compliance to HD , reduction of cardiovascular risk and global mortality. Moreover, our data may be verified in other population and clinical subset, increasing the usefulness of echocardiography for not only qualitative, but quantitative non-invasive hemodynamic evaluation.

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TABLES

Table I Clinical and Dialytic characteristics of the studied population;

Study population	
Age (years)	55.7±12.2
Sex (male)	14 (51%)
Weight (kg)	66.7±14.1
dryWeight (Kg)	63.8±13.3
dryBMI (kg/m2)	23.3±3.6
SBP (mmHg)	137.9±20.6
DBP (mmHg)	67.6±13.1
MBP (mmHg)	91.0±13.4
PP (mmHg)	70.4±18.8
HR (bpm)	74.7±12.78
Hypertension (%)	73.9%
Diabetes (%)	23%
HUF ml/h	1201.8±633.2
HCT (%)	29.5±4.9

BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, MBP: mean blood pressure, PP: pulse pressure, HR: heart rate, HUF ultrafiltration velocity; HCT: hematocrit

Table II : Baseline echocardiographic characteristics of studied population

Left ventricle	
LV systolic volume (cc)	38.9±19.5
LV diastolic volume (cc)	98.2±32.6
LVMi (g/m ²)	114.4±37.0
RWT	0.43±0.1
LVH (%)	42
Concentric (%)	27.7
Eccentric (%)	12.3
LVOT (mm)	20±2.4
EF (%)	61.6±9.1
E' sept (cm/s)	8.9±3.1
E' lat (cm/s)	10.6±3.1
E/E'	7.8±3.1
Left Atrium	
LAVi (cc/m ²)	28.2±10.7
Enlarged LAV (%)	39
Right Ventricle	
Systolic Area (cm ²)	8.5±3.4
Diastolic Area (cm ²)	17.0±5.1
TAPSE (mm)	21.5±4.5
Area change (%)	47.0±24.1

S' trc (cm/s)	10.8±2.9
E' trc (cm/s)	10.5±2.9
E/E'trc	5.4±2.07

Inferior Vena Cava

Collapsibility (%)	60.9±28.0
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LV: left ventricle; LVMi: indexed left ventricular mass; RWT: relative wall thickness; LVH: left ventricular hypertrophy; LVOT: left ventricular outflow tract; EF: Ejection fraction; E: transmitralic early diastolic wave ; E' sept: Early diastolic wave on septal mitralic annulus tissue doppler; E' lat : Early diastolic wave on lateral mitralic annulus tissue doppler; LAV: Left atrial volume; LAVi: indexed Left atrial volume; S' trc: systolic wave on tricuspidal annulus tissue doppler; E' trc : Early diastolic wave on tricuspidal annulus tissue doppler;

Table III Variation of clinical and echocardiographic parameters during hemodialysis dehydration;

	T0	T1	End	P
Clinical parameters				
SBP (mmHg)	139.1±19.5	126.1±17.8 ^a	130.0±28.2 ^a	
DPB (mmHg)	70.0±13.2	68.3±13.6	68.6±16.6	
MPB (mmHg)	93.0±12.9	87.5±13.4 ^a	89.1±19.0	
PP (mmHg)	69.0±18.6	57.8±15.0 ^a	61.3±19.8 ^a	
HR (bpm)	73.9±13.5	72.0±12.7	73.8±15.0	
Echocardiographic parameters				
Left ventricle				
LVEDd (mm)	45.3±7.6	43.1±7.3	41.8±6.7	0.001
LVEDdi (cm/m2.7)	11.9±2.7	11.2±2.6	11.2±2.6	0.04
LV diastolic volume (cc)	98.2±32.6 ^b	92.5±29.9 ^b	82.7±31.3 ^b	0.003
LV systolic volume (cc)	39.0±19.4	38.5±19.0	35.3±19.8	0.12
LVMi (g/m2)	114.4±37 ^b	104.5±35.1 ^b	95.6±30.9 ^b	<0.0001
RWT	0.43±0.09	0.43±0.09	0.44±0.09	0.5
LVOT(mm)	20.0±2.4	19.9±2.2	20.1±2.1	0.38
EF (%)	61.6±9.1	59.4±9.7	58.7±9.6 ^c	0.04
E (m/s)	0.71±0.19 ^b	0.54±0.19 ^b	0.49±0.19 ^b	<0.0001
A (m/s)	0.66±0.19 ^b	0.60±0.17 ^c	0.59±0.17 ^c	0.003
E/A	1.13±0.37 ^b	0.99±0.36 ^b	0.87±0.38 ^b	<0.0001
IVRT (ms)	94.93±32.40 ^b	118.63±37 ^c	118.38±42 ^c	<0.0001
E' sept (cm/sec)	8.9±3.1	7.6±2.0	7.5±1.7	<0.0001
E' lat (cm/sec)	10.6±3.1 ^b	9.8±3.1	9.4±3.1	0.0001
E/E'	7.8±3.1 ^c	6.3±2.4	5.8±1.8	<0.0001
Stroke work (cJ)	127.1±42.9 ^c	99.4±32.8	93.9±29.1	0.004

Left Atrium				
LAV (cc)	47.2±16.6	42.9±26.3	36.4±15.6 ^b	<0.0001
LAVi (cc/m2)	28.2±10.7	25.6±16.4	21.6±9.9 ^b	<0.0001
Inferior Vena Cava				
Collapsibility (%)	60.9±28.0 ^c	81.2±20.6	86.6±13.5	<0.0001

SBP: systolic blood pressure, DBP: diastolic blood pressure; MAP: mean blood pressure; PP: pulse pressure; HR: heart rate. ^a p < 0.05 vs T0

LV: left ventricle; LVEDd: LV end diastolic diameter ; LVEDi: LVED indexed to body height 2.7; LVMi indexed left ventricular mass; RWT: relative wall thickness; LVOT: left ventricular outflow tract; EF: Ejection fraction; E: transmitralic early diastolic wave; A : transmitralic atrial diastolic wave; IVRT: isovolumetric relaxation time; E' sept: Early diastolic wave on septal mitralic annulus tissue doppler; E' lat Early diastolic wave on lateral mitralic annulus tissue doppler LAV: Left atrial volume; LAVi: indexed Left atrial ^b vs. others; ^c vs baseline

Table IV Clinical and echocardiographic characteristics of the study population according to real percentage overload;

	Mild overload	Severe overload	P
	<4%	>4%	
Clinical parameters			
Age (years)	49.4±12.0	59.9±10.2	0.002
SBP (mmHg)	137.9±17.8	140.3±21.6	0.66
DBP(mmHg)	71.3±15.9	68.6±10.4	0.49
MAP (mmHg)	93.5±13.4	92.5±12.8	0.8
PP (mmHg)	66.6±20.7	71.7±16.8	0.34
Echocardiographic parameter			
Left Ventricle			
LVEDd (mm)	42.8±7.0	47.9±7.5	0.01
LVEDdi (cm/m2.7)	11.1±2.9	12.8±2.3	0.03
LV diastolic volume (cc)	93.4±32.7	103.5±32.7	0.34
LV systolic volume (cc)	35.2±18.4	43.1±20.3	0.21
LVMi (g/m2)	97.2±31.0	131.9±35.5	0.001
RWT	0.4±0.1	0.4±0.1	0.57
EF (%)	63.7±7.8	59.2±10.1	0.13
E (m/s)	0.73±0.21	0.69±0.18	0.5
A (m/s)	0.65±0.19	0.67±0.20	0.71
E/A	1.18±0.39	1.09±0.35	0.41
E' sept (cm/sec)	9.2±3.3	8.3±2.7	0.4
E' lat (cm/sec)	11.0±3.5	10.0±2.6	0.4
E/E'	7.8±3.8	8.0±2.7	0.86
Stroke work (cJ)	111.1±31.5	144.5±47.3	0.008

Left Atrium			
LAV (cc)	47.6±17.4	46.7±16.1	0.9
LAVi (cc/m ²)	46.7±16.1	28.4±10.2	0.86
Inferior Vena Cava			
Collapsibility (%)	61.1±25.9	59.1±30.1	0.81

SBP:systolic blood pressure; DBP:diastolic blood pressure; MAP:mean arterial pressure, PP:pulse pressure; LVEDd: left ventricular end diastolic diameter ; LVEDdi: LVED indexed to body height 2.7; LVMi indexed left ventricular mass; RWT: relative wall thickness; EF: Ejection fraction; E: transmitralic early diastolic wave; A : transmitralic atrial diastolic wave; E' sept: Early diastolic wave on septal mitralic annulus tissue doppler; E' lat Early diastolic wave on lateral mitralic annulus tissue doppler LAV: Left atrial volume; LAVi: indexed Left atrial volume

LEGEND TO FIGURES

Figure 1 : Study protocol

Figure 2: Regression analysis showing relation between percentage dehydration indexed telediastolic diameter (top) and stroke work (bottom)

Figure 1

Figure 2