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Endothelial-driven increase in plasma thrombin generation characterising a new hypercoagulable phenotype in acute heart failure

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ABSTRACT

Background: Subjects with heart failure (HF) are at higher risk of developing thrombosis. We investigated whether endothelium activation and inflammation induce a prothrombotic biological profile in patients with acute decompensated HF (ADHF) and sinus rhythm.
Methods: Our prospective study included 34 ADHF patients, 30 patients with stable chronic HF (CHF) and 30 control inpatients without HF. *In vitro* thrombin generation and its downregulation by activated protein C (APC) was monitored by calibrated automated thrombography at hospital admission, at the day of discharge and after discharge, following at least six weeks of clinical stability. Circulating endothelium-derived extracellular vesicles (eEVs) were quantified by flow cytometry and nucleosomes by ELISA.
Results: Thrombin generation is increased and APC sensitivity is decreased independently of platelets in ADHF at admission compared to controls ($p < 0.01$). Thrombin generation was also increased in CHF but only in the presence of platelets. Plasma markers of endothelium activation (von Willebrand factor, factor VIII, procoagulant eEVs and circulating nucleosomes) and the ability of plasmas to induce neutrophil extracellular trap formation in control neutrophils are elevated in ADHF at admission compared to controls ($p < 0.001$). In-hospital prothrombotic changes in ADHF improved significantly at the post-discharge time-point. Circulating nucleosomes were positively correlated with APC sensitivity ($p = 0.013$) and annexin-V-positive eEVs ($p = 0.004$).
Conclusions: This proof-of-concept study identified an endothelial-driven hypercoagulable phenotype at the acute phase of decompensated HF contrasting with the platelet-dependent prothrombotic state in CHF. These results highlighted a cross-talk between circulating eEVs and nucleosomes, procoagulant factors and impairment of the APC anticoagulant activity in ADHF.

1. Introduction

Despite the benefits observed with recent therapies including neurohormonal modulators, the prognosis of patients hospitalized for acute decompensated heart failure (ADHF) remains poor, with readmission rates approximating 50% at 6 months [1] and 1-year mortality up to 30% [2]. This doomed prognosis is partly influenced by thromboembolic events, which occur in up to 30% of patients and are often the cause of death [3]. Multiple mechanisms may contribute to a hypercoagulable state in patients with heart failure (HF) such as enhancement of soluble procoagulant factors, endothelial activation or inflammation [4, 5]. Therefore, thrombotic mechanisms may emerge as potential therapeutic targets of interest in HF [5, 6]. Targeted reduction of thrombin generation with a direct oral anticoagulant, rather than non-selective depletion of multiple vitamin K–dependent clotting factors with warfarin, may hold promise. Such an approach is currently under investigation in the COMMANDER-HF randomized trial, enrolling approximately 5000 patients with the anti-Xa rivaroxaban agent *versus* placebo initiated shortly after discharge from ADHF [6]. However, to our knowledge there has been no report of an accurate biological assessment of thrombin generation pathways in longitudinal studies of patients with ADHF.

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Endothelium exerts its function in maintaining vascular homeostasis through the balance between its natural anticoagulant function supported by the activated protein C (APC) pathway and the induced procoagulant activities. Disruption of this endothelium-dependent APC pathway promotes endocardial thrombosis in ADHF in mice [7]. Furthermore, high levels of endothelium-derived extracellular vesicles (eEVs) are independent predictors of cardiovascular events in HF patients [8]. This is also in favor of procoagulant properties of endothelium in HF.

Recently, a prothrombotic activity has been attributed to neutrophil extracellular traps (NETs), a complex network of extracellular decondensed chromatin fibres decorated with histones, granule-derived enzymes and cytoplasmic proteins. Histones that circulate as part of nucleosomes are detrimental to endothelium and promote thrombus formation [9].

We hypothesized that endothelial activation enhanced by NETs formation in ADHF promotes thrombin generation *via* the production of procoagulant eEVs and the parallel downregulation of the APC pathway. We therefore measured thrombin over the complete course of its formation and inactivation in ADHF patients using calibrated automated thrombography (CAT), including the evaluation of the APC pathway. The clinical value of such a phenotyping has been demonstrated since the amount of thrombin measured in CAT is proportional with the bleeding or thrombotic tendency [10–12]. We also focused on markers of endothelium activation and NETs in relation to their procoagulant activity.

2. Methods

2.1. Patients and controls

The study was a single center prospective study of 34 consecutive patients admitted to a cardiology department for ADHF (all in New York Heart Association NYHA functional class III or IV) defined in accordance with the European Society of Cardiology guidelines as the rapid onset/progression of HF symptoms and signs secondary to abnormal cardiac function requiring hospital admission [13]. Blood was collected at hospital admission, at the day of discharge and after hospital discharge following at least six weeks of clinical stability. Hemodynamic measurements were a routine part of clinical patient assessment and the HF therapy during hospital stay and at discharge was at the discretion of the treating physician. A group of 30 patients with stable chronic HF (CHF) recruited from outpatient clinics was also included to identify differences with the acute phase of decompensated HF.

The study included 30 control inpatients (CT) with preserved ventricular function referred to the department of cardiology for a scheduled medical assessment and who did not experience HF but had similar patterns of comorbidities, risk factors (e.g., hypertension, diabetes and smoking) and background medication to clarify the potential confounding contribution of hospital stay.

Exclusion criteria for all study groups were factors that could affect coagulation: infectious and inflammatory disorders, neoplasia, serum creatinine >200 μmol/L, use of steroid drugs, acute coronary syndrome, atrial fibrillation and all comorbidities requiring therapeutic anticoagulation. Exclusion criteria during hospital stay were worsening HF requiring assist device or heart transplant.

All ADHF patients received a venous thromboembolism (VTE) prophylaxis (enoxaparin 4000 IU once daily). Blood collection was performed before VTE prophylaxis at admission and 36 h after the last enoxaparin injection at the day of discharge. No VTE prophylaxis was administered in CT and CHF patients.

The study protocol was approved by the local ethics committee (Comité de Protection des Personnes agreement 15/10/2014). All study patients provided written informed consent.

2.2. Collection of blood samples

Venous blood samples were collected into Monovette® (Sarstedt, Nümbrecht, Germany) syringes containing 1/10 vol of 0.106 M sodium citrate. All analyses were performed within 2 h after blood collection.

Platelet-Rich Plasma (PRP) was prepared by blood centrifugation at 190*g* for 10 min at 20 °C.

The PRP was recovered and adjusted to 150 G/L for CAT by the addition of platelet-poor plasma obtained by centrifugation of the remaining blood at 1750*g* for 10 min at 20 °C. Platelet-free Plasma (PFP) was obtained from platelet-poor plasma centrifuged at 13000*g* for 30 min at 4 °C. PFP was immediately frozen at −80 °C and thawed 15 min at 37 °C when needed.

For EV enumeration and characterization, blood was subjected to two successive centrifugations at 2500*g* for 15 min and the platelet-depleted plasma (PDP) was prepared and stored at −80 °C until use.

2.3. Thrombin generation assays

CAT was performed at 37 °C using a dedicated software program (Thrombinoscope BV, Maastricht, The Netherlands) as reported previously [14]. Coagulation was triggered with 0.5 pM recombinant human tissue factor (TF) (Innovin®, Dade Behring). For measurements in PFP, a home-made mixture of phosphatidyl-choline/serine/ethanolamine (60/20/20, mol%) at a final concentration of 4 μM was added. Thrombin generation curves were recorded in triplicate in the absence or in the presence of in-house APC at several final concentrations (6.7, 13.9, 25 and 65 nM for PRP and 0.85, 1.7, 6.7 and 13.9 nM for PFP) [11]. The total amount of thrombin activity (*i.e.* the endogenous thrombin potential, ETP) was assessed as the area under the curve. The APC concentration that produced a 50% inhibition of ETP was defined as IC_{50}-APC.

2.4. Quantification of hemostatic variables and endothelial markers

All measurements were performed in PDP. Soluble thrombomodulin (TM), soluble endothelial protein C receptor (EPCR), free tissue factor pathway inhibitor (TFPI), von Willebrand factor (VWF) and factor VIII (FVIII) were measured by ELISA kits (Asserachrom, Diagnostica Stago™, Asnières, France).

2.5. Measurement of endothelium-derived extracellular vesicles

The fluorescent antibodies, CD144-phycoerythrin (CD144-PE) and allophycocyanin-EPCR were purchased from BD Pharmingen™. The other antibody against TF, clone TF9-10H10, allophycocyanin-TF was from Biotechne™, and annexin-V (AnnV)-FITC from Fisher Scientific™. EVs were enumerated by high-sensitivity flow cytometry using a standardized procedure [15]: plasma (20 μL) was incubated with the optimal concentration of specific antibody plus 10 μL of AnnV-FITC. Counting beads (20 μL) (Flow Count Fluorospheres; Beckman-Coulter™, Miami, FL, USA) were added to each sample in order to express EV counts as absolute numbers per μL of plasma. Stained samples were analyzed on a GALLIOS instrument (Beckman-Coulter™) using a calibrated bead strategy as previously described [16]. Endothelium-derived EVs were defined as CD144+ events.

2.6. Determination of nucleosome plasma levels and NET formation assay

Circulating levels of nucleosomes were measured in PDP by a specific ELISA using a pair of antibodies directed against the histone and DNA components of nucleosomes (Cell Death Detection-ELISA plus, Roche Diagnostics, Sigma-Aldrich). Because the manufacturer did not provide a standard curve, a reference material containing high amounts of endogenous nucleosomes was used and nucleosome values were reported in arbitrary units.

Peripheral blood neutrophils were isolated from healthy donors using the EasySep™ Direct Human Neutrophil Isolation Kit (StemCell). The control neutrophils were resuspended in RMPI medium without phenol red, seeded at 1.5×10^5 cells/well and incubated in duplicate with 5% plasmas from patients at 37 °C for 3 h. NETotic cells were detected by addition of 200 nM Sytox Green (Invitrogen) to each well. Extracellular DNA was quantified with a fluorometer at excitation/emission wavelengths of 485/520 nm. Background fluorescence recorded in plasmas without neutrophils was subtracted from all samples.

2.7. Statistical analysis

Categorical data are presented as numbers (%); continuous data are presented as means ± standard error (SD). Differences in categorical values were performed using Pearson's χ^2 test or non-parametric Fisher's exact test as appropriate. Continuous variables were compared using the Wilcoxon test. The variation of the studied variables in ADHF patients throughout the follow-up period was assessed by using the Friedman test. Correlations were assessed by calculating the Spearman correlation coefficient. Alpha risk was fixed to 5% for all analyses. Analyses were done using SAS 9.4 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Baseline clinical characteristics

The ADHF group at baseline was similar to the non-HF cardiology patients (CT) group with regard to the sex-ratio, comorbidities and risks factors (Table 1). ADHF had decreased left ventricular (LV) ejection fraction and increased LV end diastolic volume, pulmonary arterial pressures and LV filling pressures estimated by ratio E/e′. Serum B-type natriuretic peptide (BNP) and C-reactive protein (CRP) levels were higher in ADHF patients than in controls. No significant difference was found in the use of active cardiovascular medication except for diuretics, which were more often used in ADHF patients. Five ADHF patients died within three months after hospital discharge without available biological follow-up.

Table 1
Baseline patient's characteristics.

	CT (N = 30)	CHF (N = 30)	p-Value CT vs CHF	ADHF (N = 34)	p-Value CT vs ADHF	p-Value CHF vs ADHF	ADHF discharge (N = 34)	ADHF post-discharge (N = 13)	p-Value ADHF Ad-Dis-PostDis[a]
Demography									
Male gender	22 (73%)	23 (77%)	0.77	22 (65%)	0.46	0.30	22 (65%)	11 (85%)	
Age (years)	65 ± 15	69 ± 12	0.33	73 ± 17	0.03	0.17	73 ± 17	71 ± 13	
Risk factors									
Hypertension	18 (60%)	16 (53%)	0.60	27 (79%)	0.09	0.03	27 (79%)	11 (85%)	
Diabetes	8 (27%)	5 (17%)	0.34	15 (44%)	0.15	0.02	15 (44%)	5 (38%)	
Smoking	8 (27%)	12 (40%)	0.27	7 (21%)	0.57	0.09	7 (21%)	4 (31%)	
Previous history									
Valvular cardiopathy	4 (13%)	4 (13%)	1.00	8 (24%)	0.35	0.35	8 (24%)	3 (23%)	
Ischemic cardiopathy	14 (47%)	18 (60%)	0.30	13 (38%)	0.50	0.08	13 (38%)	5 (38%)	
Implantable cardiac defibrillator	0 (0%)	14 (47%)	<0.0001	3 (9%)	0.10	0.0006	3 (9%)	1 (8%)	
Cardiac resynchronisation therapy	0 (0%)	3 (10%)	0.24	4 (12%)	0.12	1.0	4 (12%)	1 (8%)	
Chronic kidney disease	3 (10%)	3 (10%)	1.00	9 (27%)	0.11	0.12	9 (27%)	2 (15%)	
Concomitant medications									
Beta-blockers	17 (57%)	25 (83%)	0.009	27 (79%)	0.07	0.29	27 (79%)	10 (77%)	
ACE inhibitors	17 (57%)	26 (87%)	0.003	29 (85%)	0.02	0.35	29 (85%)	12 (92%)	
Diuretics	3 (10%)	17 (57%)	<0.0001	32 (94%)	<0.0001	0.001	32 (94%)	13 (100%)	
Statins	17 (57%)	23 (77%)	0.052	22 (65%)	0.62	0.13	22 (65%)	10 (77%)	
Aspirin	22 (73%)	24 (83%)	0.35	24 (71%)	0.64	0.16	24 (71%)	8 (62%)	
Clopidogrel	7 (23%)	5 (17%)	0.56	13 (38%)	0.23	0.08	13 (38%)	4 (31%)	
Echocardiography									
LVEDV (mL)	91 ± 9	166 ± 63	0.0001	132 ± 51	0.02	0.07	132 ± 55 (17)	155 ± 60 (9)	0.04
LVESV (mL)	57 ± 10	102 ± 36	0.002	82 ± 39	0.07	0.10	85 ± 42 (17)	103 ± 46 (9)	0.37
LVEF (%)	59 ± 3	31 ± 8	<0.0001	35 ± 15	<0.0001	0.22	36 ± 12 (17)	35 ± 10 (9)	0.18
E velocity (cm/s)	86 ± 23 (26)	85 ± 30 (22)	0.90	105 ± 13 (25)	0.01	0.01	98 ± 20 (15)	66 ± 24 (10)	0.01
e′ velocity (cm/s)	12 ± 2 (26)	9 ± 4 (22)	0.0003	6 ± 2 (25)	<0.0001	0.01	8 ± 5 (15)	8 ± 3 (10)	0.26
E/e′ ratio			0.52		0.35	0.55			0.57
<8	16 (26)	7 (22)		0 (25)			4 (15)	5 (10)	
8–14	10 (26)	9 (22)		6 (25)			6 (15)	3 (10)	
>14	0 (26)	6 (22)		19 (25)			5 (15)	2 (10)	
sPAP (mm Hg)	33 ± 4	44 ± 7	<0.0001	45 ± 10	<0.0001	0.76	39 ± 5 (9)	39 ± 6 (17)	0.01
Biology at admission									
BNP (ng/L)	101 ± 11	549 ± 1015	<0.0001	1432 ± 1468	<0.0001	<0.0001	921 ± 1155	267 ± 120	<0.0001
Creatinine (mg/L)	11.4 ± 0.5	10.9 ± 0.5	0.21	13.2 ± 1.0	0.50	0.19	ND	ND	
GFR (mL/min/1.73 m^2)	69 ± 14	57 ± 12	0.0002	55 ± 19	0.002	0.50	ND	ND	
Glycemia (g/L)	1.0 ± 0.2	1.1 ± 0.3	0.34	1.2 ± 0.4	0.03	0.29	ND	ND	
CRP (mg/L)	5.6 ± 2.9	7.2 ± 6.5	0.28	14.9 ± 10.6	<0.0001	<0.0001	ND	ND	
ASAT (UI/L)	19 ± 5	22 ± 20	0.0004	36 ± 34	0.24	0.002	ND	ND	
ALAT (UI/L)	17 ± 5	19 ± 13	0.43	38 ± 63	0.09	0.03	ND	ND	
Fibrinogen (g/L)	2.7 ± 0.7	2.5 ± 0.8	0.42	2.9 ± 1.1	0.38	0.15	ND	ND	
Leukocyte count ($10^3/mm^3$)	6.4 ± 1.9	6.4 ± 1.6	0.52	6.8 ± 2.2	0.94	0.44	ND	ND	
Platelet count ($10^3/mm^3$)	215 ± 107	197 ± 107	0.57	201 ± 81	0.52	0.86	ND	ND	

Results are mean ± SD or frequency (percent). ACE indicates angiotensin-converting enzyme; LVEDV, left ventricular end diastolic volume; LVESV, left ventricular end systolic volume; LVEF, left ventricular ejection fraction; sPAP, pulmonary arterial pressures; BNP, B-type natriuretic peptide; GFR, glomerular filtration rate; CRP, C-reactive protein; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; ND, not determined. When the full data for the patients is not available the numbers of patients is given in brackets.
[a] Friedman test for quantitative variables throughout the follow-up period.

Control CHF patients differed substantially from ADHF patients with respect to hypertension and diabetes, which were more frequent in ADHF patients. As expected, levels of BNP and CRP were higher in ADHF patients than in CHF patients. Patients with CHF and ADHF presented similar reduced kidney function, as measured by GFR (<60 mL/min), compared with controls. Leukocyte and platelet counts as well as fibrinogen levels were not different in all groups. Echocardiographic parameters including LV filling pressures estimated by ratio E/e′ were consistent with inclusion criteria of each group (Table 1).

3.2. In vitro thrombin generation is accelerated and increased in ADHF patients at hospital admission

At hospital admission, ETP in the presence of platelets (PRP adjusted at the same platelet count) was higher whereas lag-time and time to peak were markedly but not significantly shorter for ADHF patients compared to controls (Fig. 1A and Table 2). In absence of platelets (PFP), thrombin peak, ETP and velocity were increased and lag-time and time to peak were shortened for ADHF patients compared to controls (Fig. 1B and Table 2).

Compared to controls, ETP was increased in CHF patients only in the presence of platelets. There was no significant difference in ETP between CHF and ADHF patients at admission in the presence of platelets whereas ETP was higher in the absence of platelets of ADHF patients.

Increase in ETP and/or thrombin peak values was maintained on the day of discharge but was lost at the post-discharge time-point (Fig. 1A and B and Table 2).

3.3. APC sensitivity is impaired in ADHF patients at hospital admission

ETP in the presence of APC was consistently higher at hospital admission compared to controls (Fig. 1C and D). The APC concentration (IC_{50}-APC) that gave half maximum inhibition of ETP without APC was significantly increased in the presence of platelets at hospital admission compared to controls (Table 2). The IC_{50}-APC in the absence of platelets tended to increase in ADHF patients as compared to controls but did not

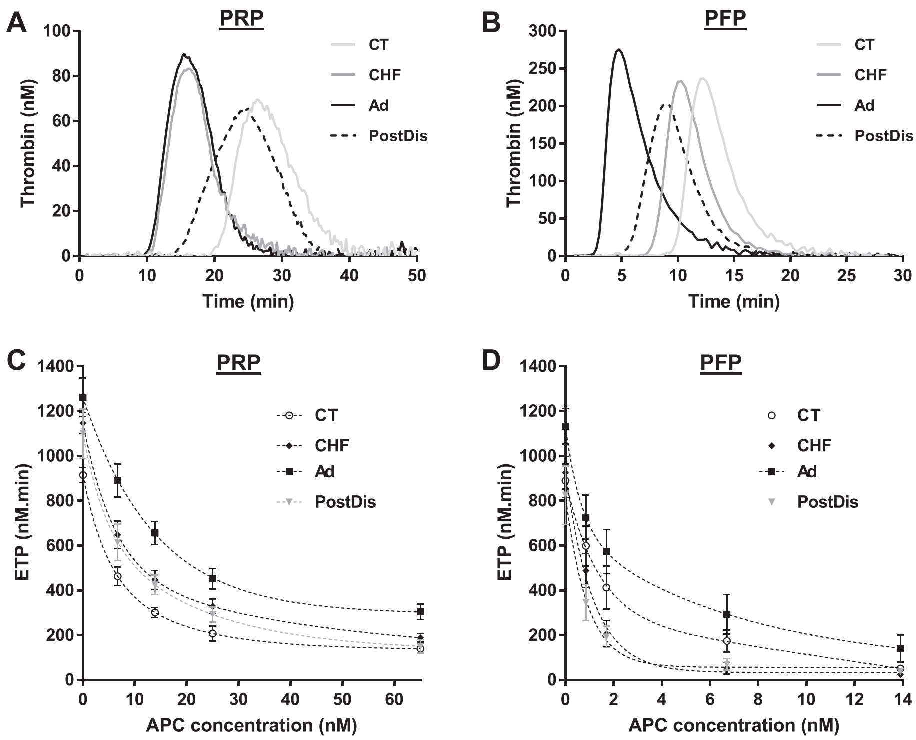

Fig. 1. Acute heart failure increases thrombin generation and impairs activated protein C sensitivity. Thrombin generation was triggered by 0.5 pM tissue factor and monitored with the calibrated automated thrombography assay. A and B) Representative thrombin generation curves in platelet-rich plasma (PRP) adjusted to 150×10^9 platelets/L or in platelet-free plasma (PFP) + 4 μM phosphatidyl-serine/-ethanolamine/-choline vesicles respectively. C and D) Inhibition of endogenous thrombin potential (ETP) by activated protein C (APC) in PRP or in PFP + 4 μM phosphatidyl-serine/-ethanolamine/-choline vesicles respectively. CT, control non-heart failure cardiology patient; CHF, CHF patient; Ad, ADHF patient at hospital admission; PostDis, ADHF patient at the post-discharge time-point.

Table 2
Thrombin generation parameters and circulating markers of endothelial activation.

	CT	CHF	p-Value CT vs CHF	ADHF admission	p-Value CT vs ADHF Ad	p-Value CHF vs ADHF Ad	ADHF discharge	ADHF post-discharge	p-Value ADHF Ad-Dis-PostDis[a]
Platelet-rich plasma (PRP)									
N	30	30		32			27	13	
ETP (nM/min)	983 ± 162	1147 ± 260	0.007	1293 ± 463	0.009	0.25	1375 ± 451	1100 ± 398	0.053
Lag-time (min)	17.1 ± 7.3	12.7 ± 4.1	0.02	13.6 ± 6.4	0.054	0.50	13.7 ± 3.6	15.4 ± 4.2	0.33
Thrombin peak (nM)	81 ± 44	100 ± 65	0.44	100 ± 58	0.16	0.72	112 ± 53	68 ± 38	0.007
Time to peak (min)	27.7 ± 12.9	21.7 ± 8.2	0.07	21.4 ± 8.4	0.08	0.97	21.3 ± 6.0	26.7 ± 8.0	0.03
Velocity (nM/min)	16.2 ± 18.2	17.7 ± 18.1	0.89	16.9 ± 14.4	0.42	0.61	18.0 ± 12.0	7.9 ± 6.6	0.02
IC_{50}-APC (nM)	6.9 ± 3.2	8.9 ± 5.0	0.14	14.3 ± 13.7	0.008	0.23	9.8 ± 5.5	8.0 ± 4.0	0.11
ETP × IC_{50}-APC	6956 ± 3862	10,174 ± 6232	0.058	18,460 ± 19,555	0.0002	0.06	13,891 ± 9917	8051 ± 4848	0.03
Platelet-free plasma (PFP)									
N	29	30		28			29	13	
ETP (nM/min)	806 ± 331	932 ± 385	0.28	1150 ± 412	0.004	0.08	1179 ± 427	900 ± 510	0.03
Lag-time (min)	10.9 ± 6.4	8.4 ± 3.6	0.16	3.9 ± 1.3	0.0001	<0.0001	4.7 ± 1.2	5.0 ± 2.0	0.006
Thrombin peak (nM)	149 ± 103	192 ± 105	0.12	237 ± 98	0.003	0.12	229 ± 105	169 ± 85	0.43
Time to peak (min)	13.3 ± 6.1	11.4 ± 4.0	0.25	6.5 ± 1.5	<0.0001	<0.0001	7.6 ± 2.0	7.9 ± 2.1	0.006
Velocity (nM/min)	66.1 ± 69.2	85.0 ± 64.9	0.11	107.8 ± 64.5	0.005	0.11	99.1 ± 64.0	61.8 ± 33.0	0.43
IC_{50}-APC (nM)	0.8 ± 0.6	0.7 ± 0.6	0.53	2.3 ± 3.5	0.12	0.03	1.4 ± 2.7	0.6 ± 0.5	0.058
ETP × IC_{50}-APC	781 ± 808	604 ± 591	0.39	3133 ± 5503	0.03	0.004	1901 ± 3892	434 ± 448	0.02
Circulating markers of endothelial activation									
N	30	28		27			27	11	
sEPCR (ng/mL)	162 ± 79	168 ± 63	0.39	245 ± 83	0.0003	0.0003	239 ± 89	169 ± 46	0.048
sTM (pg/mL)	157 ± 35	163 ± 58	0.72	284 ± 100	<0.0001	<0.0001	274 ± 114	146 ± 27	0.0002
Free TFPI (ng/mL)	10.9 ± 2.5	11.9 ± 2.8	0.16	13.9 ± 4.1	0.002	0.055	12.8 ± 3.3	12.3 ± 2.4	0.62
FVIII/VWF ratio	0.46 ± 0.26	0.65 ± 0.21	0.001	0.46 ± 0.25	0.91	0.002	0.32 ± 0.14	0.42 ± 0.16	0.48
Endothelium-derived extracellular vesicles									
N	30	22		32			33	13	
$AnnV^+/TF^+$ (EVs/μL)	57 ± 44	85 ± 76	0.36	258 ± 321	0.003	0.045	116 ± 116	61 ± 49	0.12
$EPCR^+$ (EVs/μL)	87 ± 54	104 ± 92	0.99	128 ± 153	0.72	0.66	122 ± 122	98 ± 76	0.48
$EPCR^+/TF^+$ (EVs/μL)	69 ± 54	55 ± 44	0.34	116 ± 151	0.24	0.07	107 ± 114	64 ± 33	0.27

Values are means ± SD. ETP indicates the endogenous thrombin potential in the absence of activated protein C (APC); IC_{50}-APC, the APC concentration needed to reduce ETP by 50%; EPCR, endothelial protein C receptor; TM, thrombomodulin; VWF, von Willebrand factor; FVIII, coagulation factor VIII; TFPI, tissue factor pathway inhibitor; EVs extracellular vesicles, AnnV, annexin V; TF, tissue factor.

[a] Friedman test for quantitative variables throughout the follow-up period.

reach statistical significance. Level of CRP at hospital admission was positively correlated with IC_{50}-APC in the absence of platelets. No differences were found between CHF patients and controls or ADHF patients at admission in the presence of platelets whereas IC_{50}-APC in the absence of platelets was increased in ADHF compared to CHF patients.

When ETP (without APC) and IC_{50}-APC are combined using their arithmetic product as previously reported [17], a procoagulant phenotype was confirmed by a near threefold value at hospital admission compared to the controls (Table 2). This parameter tended to increase in PRP of CHF patients as compared to controls but did not reach statistical significance. Compared to CHF patients, ETP × IC_{50}-APC values were increased in the absence of platelets of ADHF patients at admission.

ETP values in the presence of APC were shifted downwards on the post-discharge time-point compared to admission (Fig. 1C and D). At hospital discharge, and even more so at the post-discharge time-point, the arithmetic product ETP × IC_{50}-APC was decreased.

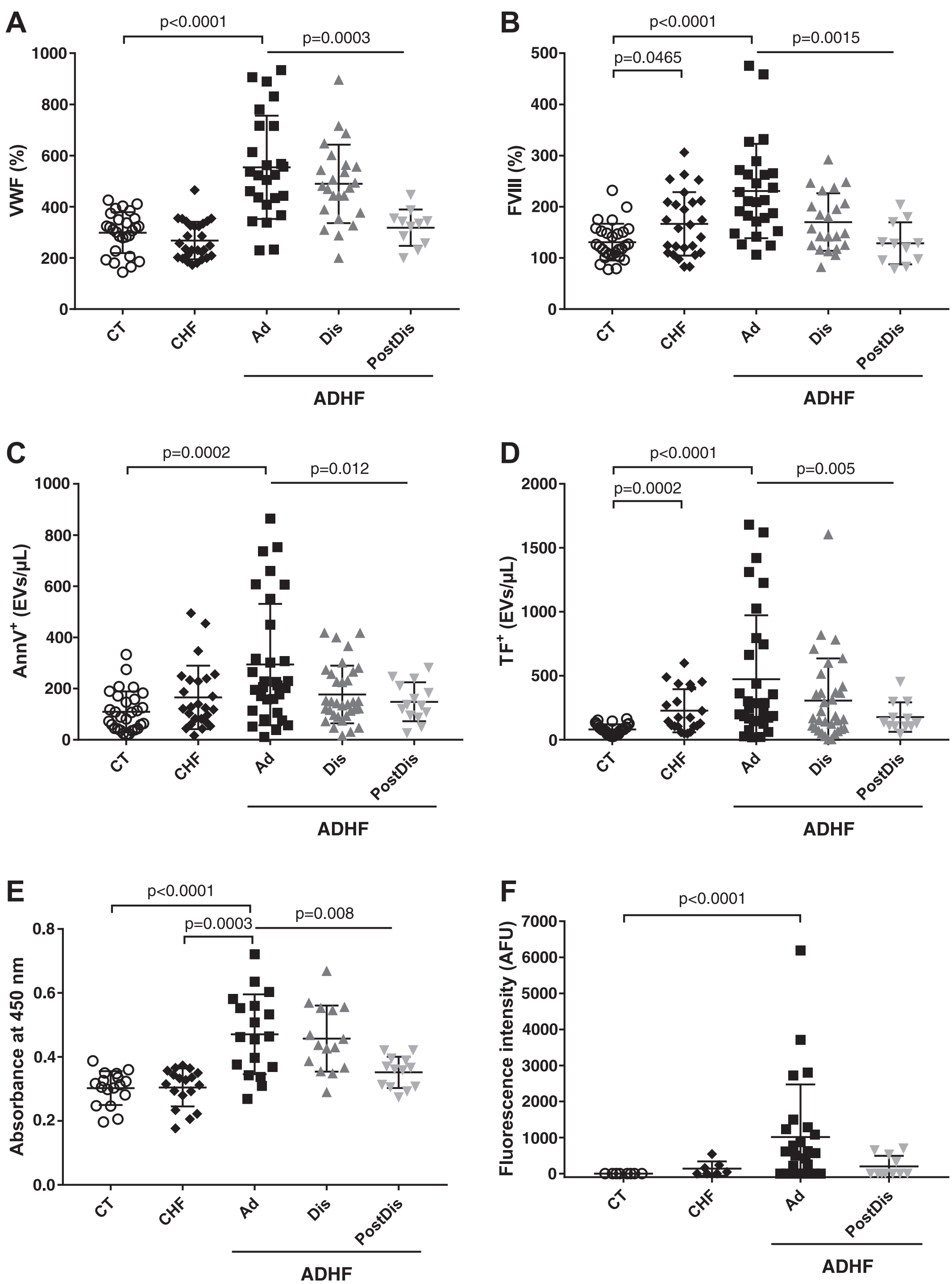

Fig. 2. Markers of endothelial activation and circulating nucleosomes are increased in ADHF patients at hospital admission. Levels of VWF (A), FVIII (B) and DNA complexed with histones (E) were measured in plasma by ELISA. Endothelium-derived extracellular vesicles (eEVs) were enumerated by flow cytometry (C and D). The ability of plasmas from patients to induce NET formation in control neutrophils was assessed by a fluorescence plate assay (F). Results are expressed in arbitrary fluorescence units (AFU). Each dot within vertical scatter plots represents a single patient; the mean ± SEM is depicted. CT, control non-heart failure cardiology patient; CHF, CHF patient; Ad, ADHF patient at hospital admission; Dis, ADHF patient at the day of discharge; PostDis, ADHF patient at the post-discharge time-point.

3.4. Soluble markers of endothelial activation are elevated at the acute decompensated phases of HF

ADHF patients had higher plasma levels of sEPCR, sTM, VWF, free TFPI and FVIII at hospital admission *versus* controls (Fig. 2 and Table 2). The increase in FVIII paralleled the increase in VWF as revealed by similar values of the FVIII/VWF ratio. Levels of these factors were significantly higher at admission of ADHF patients compared with plasma levels in the CHF group. Only FVIII and thus FVIII/VWF ratio was elevated in CHF patients compared to controls.

Elevation in these factors was maintained on the day of discharge but was lost on the post-discharge time-point except for free TFPI.

Compared to controls, the concentrations of $AnnV^+$ eEVs, TF^+ eEVs and $AnnV^+/TF^+$ eEVs was significantly higher in ADHF patients at admission (Fig. 2 and Table 2). The concentration of TF^+ eEVs in CHF patients significantly increased compared to controls. No difference in the concentrations of $EPCR^+$ eEVs and $AnnV^+/EPCR^+$ eEVs was denoted between ADHF patients and controls or CHF patients (Table 2).

The concentration of $AnnV^+$ eEVs and TF^+ eEVs in ADHF patients significantly decreased over the hospital course and was reduced on the post-discharge time-point compared to admission.

3.5. Circulating nucleosomes and the ability to induce NET formation in control neutrophils are elevated in ADHF patients at hospital admission

We measured the plasma levels of nucleosomes in a subset of ADHF patients including all those followed up after discharge. Circulating nucleosomes were increased in ADHF patients at admission compared with controls and CHF patients (Fig. 2). At the day of discharge, levels of nucleosomes remained elevated whereas a significant decline was evident on the post-discharge time-point.

Nucleosome levels at hospital admission were significantly correlated with IC_{50}-APC and ETP × IC_{50}-APC in the presence of platelets ($r = 0.59$; $p = 0.013$ and $r = 0.63$; $p = 0.006$ respectively) and $AnnV^+$ eEVs ($r = 0.76$; $p = 0.0004$).

We also evaluated the ability of patients' plasmas to induce NET formation in control neutrophils. Plasma from ADHF patients at admission showed increased capacity to induce NET formation in unstimulated neutrophils compared with plasmas from controls and CHF patients. This characteristic was lost on the post-discharge time-point.

4. Discussion

The present study demonstrates a significant shift towards a prothrombotic biological profile at the acute phase of decompensated HF. Both an increase in procoagulant properties of activated endothelium and an impairment in the downregulation of thrombin generation by the endothelium APC-EPCR anticoagulant axis associated with increased levels of circulating nucleosomes appear to represent mechanisms contributing to this hypercoagulable state in ADHF.

Thrombin generation in the presence of APC was higher for ADHF patients at hospital admission compared to controls, suggesting a defect in the anticoagulant APC pathway during the acute phase, as clearly emphasized by the markedly elevated summary index ETP × IC_{50}-APC [17]. In other pathologies, such as antiphospholipid syndrome, sepsis or idiopathic pulmonary hypertension, attenuation of the APC pathway was demonstrated to underlie the procoagulant phenotype associated with endothelial activation or inflammation [11, 18, 19]. However, these studies do not extend beyond the description of a phenotype and do not address potential mechanisms.

In our study, the prothrombotic profile observed both in the presence (PRP) and the absence (PFP) of platelets in ADHF patients suggests that platelet activation plays a modest role in the procoagulant phenotype. By contrast, this argues for a main contribution of soluble factors, especially factors related to endothelial activation.

When comparing CHF patients with controls, we also observed an increase in thrombin generation in PRP in the absence of APC, consistent with previous studies reporting a degree of hypercoagulability in chronic HF [20]. However, APC resistance was not observed in CHF patients and the prothrombotic profile was present only in the presence of platelets. This points to a differential involvement of platelets and endothelium in the hypercoagulable phenotype of CHF and ADHF patients. Our results suggest a platelet-mediated effect in the increased thrombin generation in CHF patients in contrast to ADHF. Although ASAT were significantly elevated in some CHF patients, the fact that coagulation parameters were normal, added to the evidence of this platelet-mediated effect, rules out a significant impact of liver functionality in this coagulation dysregulation.

For ADHF patients, the data gathered in our study point to a hypercoagulable state at the acute phase of decompensated HF mainly driven by endothelial activation and nucleosome release, as evidenced by a number of parameters.

First, it is known that endothelial activation results in the shedding of receptors such as TM and EPCR, both involved in the APC pathway. In their soluble circulating form, these receptors can act as antagonists of the physiological anticoagulant properties of protein C (PC) [21]. Indeed, soluble TM has been shown to be elevated in acute CHF, thus enhancing the prothrombotic state [22]. The increase in circulating sTM and sEPCR that we measure in ADHF patients is therefore likely to contribute to the disruption of the APC pathway in these patients.

The main determinant of the thrombin generation curve is FVIII [23]. It was therefore logical to measure plasma concentration of FVIII as well as its carrier protein, VWF which interestingly enough is an acute phase protein released upon endothelial activation [24]. Both proteins were significantly elevated in ADHF patients and by calculating the FVIII/VWF ratio we further confirmed that FVIII was raised as a result of VWF increase. These data point to endothelial activation as primary contributor of both VWF and FVIII and are consistent with the observation that high levels of VWF are correlated with functional class and adverse outcome in HF [22, 25].

Among additional parameters potentially contributing to the excessive thrombin generation measured in ADHF, $AnnV^+$ EV production should not be overlooked. Indeed, such production has been associated with the severity of functional status in acute HF [26]. In our study, we observed an increase in $AnnV^+$ EVs and TF^+ EVs derived from activated endothelium at hospital admission. It is therefore likely that a sudden and massive release of procoagulant eEVs would favor the assembly of procoagulant complexes over APC-dependent anticoagulant reactions due to low avidity of APC for phospholipid membrane. Since $AnnV^+/EPCR^+$ eEVs were not elevated, we can postulate that this source of membrane EPCR is not sufficient to compensate for the impairment of the APC pathway by sEPCR. Hypercoagulability is self-maintained as thrombin induces eEVs release [27] and in turn, these eEVs initiate an amplification loop of thrombin generation that locally increases thrombin action [28].

NETs also play a key role in hypercoagulability as recently described for several clinical conditions [9]. A significant correlation between thrombin generation and NET-related variables has just been highlighted in Cushing disease [29]. We report for the first time an increased level of circulating nucleosomes and a parallel increase in the ability of plasmas from ADHF patients at admission to induce NET formation in control unstimulated neutrophils. Our data uncovered a significant positive correlation between the level of circulating nucleosomes and both the sensitivity to APC and the overall thrombin-generating capacity in PRP of ADHF patients in CAT experiments. TFPI cleavage by serine proteases represents an additional mechanism to explain the contribution of NETs to thrombogenesis. The relatively modest increase in free TFPI plasma levels in our study may result from elastase-mediated cleavage of TFPI.

Beyond assessing the mechanisms underlying the endothelial-driven hypercoagulability in ADHF patients, one of the main interests in our study was the longitudinal follow-up of the patients. Our findings demonstrate for the first time that thrombin generation parameters, endothelium activation soluble markers, eEV signature and nucleosomes were sensitive to the temporal evolution of this disease. Although the hypercoagulable profile remained present over the hospital course, the prothrombotic changes were attenuated on the day of hospital discharge and were not evidenced at the post-discharge time-point. Reduction of circulating nucleosomes at the post-discharge period likely plays a role in inflammation resolution and improvement of endothelial function.

Study limitations

This study enrolls a relatively small number of ADHF patients. However, this population has the highest event rate and is in direct need of novel therapies. Furthermore, the longitudinal design of our study allows for a clear demonstration of an increased thrombin-generating capacity in the integrative phenotyping of the clotting system at the acute phase of decompensated HF. ADHF is an acute inflammatory condition as indicated by elevated BNP and CRP levels and thus it may not be specific for endothelial inflammation alone. Since most of ADHF patients received antiplatelet drugs, the level of thrombin generation might have been slightly underestimated because of their action on platelet function and also endothelial inflammation. The potential direct influence of VTE prophylaxis on thrombin generation was not evaluated in our study since CAT was performed either before VTE prophylaxis or 36 h after the last administration.

5. Conclusions

ADHF patients display an endothelial shift to a prothrombotic profile, which is characterized by an increase in procoagulant eEVs and FVIII levels promoting accelerated and increased thrombin generation and impaired down-regulation by the anticoagulant APC. We also identify elevated circulating nucleosomes as a new clue contributing to hypercoagulability. This hypercoagulable biological phenotype is specific of the acute phase of decompensated HF since it disappears after discharge in patients recovering from an acute event.

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Disclosures

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