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The Association of Subclinical Hypothyroidism and Pattern of Circulating Endothelial-Derived Microparticles Among Chronic Heart Failure Patients

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Background: Subclinical hypothyroidism (SH) is diagnosed biochemically by the presence of normal serum free thyroxine concentration, in conjunction with an elevated serum thyroid-stimulating hormone level. Recent studies have demonstrated the frequent association between SH and cardiovascular diseases and risk factors.

Objectives: To evaluate the impact of SH on patterns of circulating endothelial-derived microparticles, (EMPs) among chronic heart failure (CHF) patients

Patients and Methods: This is a retrospective study involving a cohort of 388 patients with CHF. Fifty-three CHF subjects had SH and 335 patients were free from thyroid dysfunction. Circulating levels of N-terminal-pro brain natriuretic peptide (NT-proBNP), high-sensitivity C-reactive protein (hs-CRP), thyroid-stimulating hormone (TSH), total and free thyroxine (T4), and triiodothyronine (T3), and endothelial apoptotic microparticles (EMPs), were measured at baseline. SH was defined, according to contemporary clinical guidelines, as a biochemical state associated with an elevated serum TSH level of greater 10 μU/L and normal basal free T3 and T4 concentrations.

Results: Circulating CD31+/annexin V+ EMPs were higher in patients with SH compared to those without SH. In contrast, activated CD62E+ EMP numbers were not significantly different between both patient cohorts. Using uni (bi) variate and multivariate age- and gender-adjusted regression analysis, we found several predictors that affected the increase of the CD31+/annexin V+ to CD62E+ ratio in the patient study population. The independent impact of TSH per 6.5 μU/L (odds ratio [OR] = 1.23, P = 0.001), SH (OR = 1.22, P = 0.001), NT-proBNP (OR = 1.19, P = 0.001), NYHA class (OR = 1.09, P = 0.001), hs-CRP per 4.50 mg/L (OR = 1.05, P = 0.001), dyslipidemia (OR = 1.06, P = 0.001), serum uric acid per 9.5 mmol/L (OR = 1.04, P = 0.022) on the increase in the CD31+/annexin V+ to CD62E+ ratio, was determined.

Conclusions: We believe that the SH state in CHF patients may be associated with the impaired pattern of circulating EMPs, with the predominantly increased number of apoptotic-derived microparticles.

Keywords: Chronic Heart Failure; Microparticles; Thyroid Dysfunction

1. Background

Subclinical hypothyroidism (SH) is diagnosed biochemically by the presence of normal serum free thyroxine (T4) concentration, in conjunction with an elevated serum thyroid-stimulating hormone (TSH) level (1). Recent studies have reported multiple etiologies for SH among non-pregnant females and adult males, as well as frequent associations with cardiovascular (CV) diseases and risk factors (2, 3). The strong independent association with CV diseases and chronic heart failure (CHF) indicates that SH may be a population risk factor for these conditions (4-7). Moreover, SH may be directly associated with endothelial dysfunction and impaired coronary flow reserve through specific molecular pathways in endothelial cells, by affecting NO production and facilitating the increased degradation of vasodepressor intermediates (8). Interestingly, the role of SH in cardiovascular morbidity and mortality is controversial (9). Since SH is relatively common in older patients, conflicting results on the age-related association between SH and CV risk factors and events have been reported (10-12). Although total mortality did not increase among SH subjects, the severity of SH is attributed to the elevated serum TSH level and is closely associated with CV outcomes and mortality in the adult patient population (13-15). Overall, SH may contribute to CV risk and disease development through endothelial dysfunction. In this context, circulating endothelial-derived microparticles (EMPs) may function as novel biological markers for endothelial injury, vascular tone disorders, and vascular aging (16, 17), which may demonstrate the impact of SH in CV disease progression.

EMPs are defined as a heterogeneous population of vesicles (100 - 1000 nm in diameter) that are released by cellular vesiculation and fission of the endothelial cell membrane (18). The biological effects of EMPs may be mediated by supporting cell-to-cell cross-talking because EMPs transport miRNA, active molecules, hormones, peptides, regulator proteins, etc. (19). EMPs are derived from activated or apoptotic endothelial cells and may play a pivotal role in endothelial reparation, tissue injury, and vascular remodeling (20). The different patterns of circulating EMPs in CV diseases including CHF, suggest that impaired EMP phenotypes are potentially available for risk stratification in CV and metabolic disease subjects (21-24). However, the causal role of EMP patterns in CHF patients with SH is still unclear.

2. Objectives

To evaluate the relationship between SH and the patterns of circulating EMPs in CHF patients.

3. Patients and Methods

3.1. Study Population

This is a retrospective study involving a cohort of 388 patients with documented ischemia- induced CHF who underwent angiography or PCI between April 2010 and June 2014, as well as post-myocardial infarction subjects with left ventricular ejection fractions (LVEF) of less than 45%. Sample size was calculated by using the single population proportion formula after assuming 50% prevalence and considering 95% confidence level of significance with an alpha of 0.05 (1.96), and 5% margin of error, resulting in a sample size of 388.

All these patients were selected from 1427 available patients, according to our inclusion (documented ischemia-induced CHF and LVEF < 45%) and exclusion criteria. One hundred fifty-five subjects were excluded due to non-compliance to the study protocol, in the absence of documented evidence of ischemic heart disease. Ischemic heart disease was determined when existing myocardial infarction and/or stenosis of coronary arteries (> 50% in at least one coronary artery) were documented. Among 1272 discharge reports, we utilized data from 388 patients with documented ischemia-induced CHF with LVEF < 45%. Patients with severe kidney and liver diseases, malignancy, creatinine plasma level above 440 μmol/L, estimated GFR index < 35 mL/min/M^2, brain injury within 3 months prior to study enrollment, pulmonary edema, tachyarrhythmia, valvular heart disease, thyrotoxicosis, ischemic stroke, intracranial hemorrhage, acute infections, surgery, trauma, ischemic events within the previous 3 months, inflammatory conditions within the previous month, who are currently pregnant, have implanted pacemakers, any disorder that may lead the patient to discontinue study participation, as assessed by the investigators, were excluded from the study. Among the enrolled subjects, fifty-three CHF subjects had SH and 335 were free from thyroid dysfunction.

The Zaporozhye State Medical University ethics committee review board approved the study protocol (#3 12/02/2012). The study complied with the Declaration of Helsinki and voluntary informed written consent was obtained from all patients who were included in this study.

3.2. Methods for Visualization of Coronary Arteries

Multispiral contrast-enhanced computed tomography angiography or conventional angiography was performed on all the patients prior to their inclusion in the study. The coronary artery wall structure was measured by contrast-enhanced spiral computed tomography angiography on Optima CT660 scanner (GE, USA) (25) using non-ionic contrast Omnipaque (Amersham Health, Ireland).

3.3. Echocardiography and Tissue Doppler Imaging

Transthoracic B-mode echocardiography and tissue Doppler imaging were performed according to a conventional procedure on ACUSON scanner (SIEMENS, Germany), using a phased transducer of 5 MHz. Left ventricular end-diastolic and end-systolic volumes, and LVEF were measured by the modified Simpson method (26).

3.4. Glomerular Filtration Rate Measurement

The glomerular filtration rate (GFR) was calculated using the CKD-EPI formula (27).

3.5. Biomarker Determination

All biomarkers were determined at baseline and in duplicate. In order to measure biological marker concentrations, blood samples were drawn in the morning (between 7 - 8 a.m.) and stored in cooled silicone test tubes. Samples were processed according to the manufacturers' recommendations and were centrifuged upon permanent cooling at 6,000 rpm for 3 minutes. The plasma was refrigerated immediately for storage at a temperature of -70°C until measurement.

Circulating NT-pro-BNP, high-sensitivity C-reactive protein (hs-CRP), thyroid stimulating hormone (TSH), total and free thyroxine (T4), total and free triiodothyronine (T3), were measured using the immune electrochemiluminescence technique on the AU640 analyzer manufactured by the Diagnostic Systems Group (Japan).

Concentrations of total cholesterol (TC) and cholesterol of high-density lipoproteins (HDL-C), low-density lipoproteins (LDL-C) were measured by direct enzymatic method. Serum samples (100 μL) were assayed in parallel to known standard concentrations for each biological marker. The mean interassay and intraassay coefficients of variations were calculated by conventional method (28) and were < 10% in all cases.

3.6. Determination of Subclinical Hypothyroidism

SH was defined according to contemporary clinical guidelines as a biochemical state associated with elevated serum TSH levels of > 10 μU/L and normal basal free T3 and T4 concentrations. (29).

3.7. Endothelial-Derived Apoptotic and Activated Microparticles Determination

Endothelial-derived apoptotic and activated microparticles were phenotyped by flow cytometry using the phycoerythrin (PE)-conjugated monoclonal antibody against CD31 (platelet endothelial cell adhesion molecule [PECAM]-1), CD144 (vascular endothelial [VE]-cadherin), CD62E (E-selectin), and annexin V (BD Biosciences, USA), followed by incubation with fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Biosciences, USA) per HD-FACS (High-Definition Fluorescence Activated Cell Sorter) methodology. The samples were incubated in the dark for 15 minutes at room temperature, according to the manufacturer's instructions. The samples were then analyzed on a FC500 flow cytometer (Beckman Coulter). For determination of annexin V+ EMPs, 400 μL of annexin-V binding buffer was added. For each sample, 500 thousand events were analyzed. The EMP gate was defined by size, using 0.8 and 1.1 mm beads (Sigma, St Louis, MO, USA). The CD31+/annexin V+ and CD144+/CD31+/annexin V+ microparticles were defined as apoptotic EMPs. EMPs that were positively labeled for CD62E+ were determined as EMPs that were produced from the activation of endothelial cells. Therefore, double-positive EMPs (CD31 and CD144) and triple-positive (CD144+/CD31+/annexin V+) were defined as the most specific EMPs (30, 31).

3.8. Statistical Analysis

IBM SPSS Statistics 20 for Windows (IBM Inc., Armonk, NY, USA) and GraphPad Prism for Windows, Version 5 (GraphPad Software Inc, La Jolla, CA, USA), were used for all analyses. The data were presented as mean (M) and standard deviation (± SD) or 95% confidence interval (CI), median (Me) and interquartile range (IQR), as well as numbers (n) and frequencies (%) for categorical variables. In order to compare the main parameters of the patients' groups (subject to the type of distribution of the parameters analyzed), either a two-tailed Student t-test or Mann-Whitney U-test was used, depending on data distribution, as determined by the D'Agostino-Pearson omnibus normality test. In order to compare categorical variables between groups, the Chi^2 (χ^2) and Fisher F exact tests were used. The factors, which could be potentially associated with the CD31+/annexin V+ to CD62E+ ratio, were determined by uni(bi)-variate and multi-vatriate regression analysis. A calculated difference of $P < 0.05$ was considered statistically significant.

4. Results

4.1. The Study Patient Population

The mean age of the patient population was 58.34 ± 9.60 years old. Majority of the CHF patients were moderate to severely symptomatic, had CV risk factors (hypertension, dyslipidemia) and clinically significant co-morbidities, such as T2DM (37.6%) and obesity (44.3%) (Table 1). Fifty-five CHF patients were identified as SH subjects with the discovery of elevated TSH levels < 10 μU/L along with normal ranges of T3 and T4 levels. There were no significant differences in age, male sex, hypertension and T2DM, BMI, systolic and diastolic blood pressure, heart rate, LVEF, GFR between the cohort of patients with SH and those without SH. However, there were more SH patients ($P < 0.001$ for all cases) with NYHA class III and IV CHF and dyslipedemia compared to patients without SH. There were no significant differences in serum creatinine, hemoglobin, fasting glucose, and HbA1c (Table 2) between the two cohorts. Conversely, obesity was more common among the patients without SH ($P < 0.001$). Serum total cholesterol and low-density lipoprotein cholesterol levels were higher ($P = 0.047$ and $P = 0.046$ respectively) while high-density lipoprotein cholesterol was lower ($P = 0.044$) in patients compared to those without SH, respectively. Similarly, circulating levels of NT-pro-BNP and hs-CRP were significantly elevated in patients ($P = 0.036$ for both cases) with and not in those without SH, respectively.

4.2. Treatment Approaches Among CHF Patients

All patients were treated according current clinical guidelines for the management of CHF. There were no significant differences in treatment strategy for both cohorts of CHF patients enrolled in the study, with the exception of statin therapy (Table 3). Statins were frequently used in patients with compared to those without SH ($P = 0.012$).

4.3. Pattern of Circulating Microparticles in CHF Patients

As shown in Figure 1 A, the total number of CD144+/annexin V+ phenotyped EMPs did not differ between both patient cohorts ($P = 0.22$). The double-positive (for CD144 and CD31-staining) EMP phenotype (Figure 1 B) and triple-positive EMPs (labeled as CD144+/ CD31+/annexin V+ subset; Figure 1 C) were not significantly elevated in both patient cohorts ($P = 0.26$). Mean circulating apoptotic-derived EMP numbers (CD31+/annexin V+) isolated from the peripheral blood was significantly higher in patients with compared to those without SH, respectively ($P < 0.001$; Figure 1 D). In contrast, the number of activated CD62E+ EMPs was not significantly different between both patient cohorts ($P = 0.46$; Figure 1 E). Therefore, calculated CD31+/annexin V+ to CD62E+ ratio was significantly higher in patients with compared to those without SH, respectively ($P < 0.001$; Figure 1 F).

Table 1. Demographics and Main Characteristics of Study Participants [a,b]

Variables	Entire CHF Patient Cohort (n = 388)	SH Subjects (n = 53)	None-SH Subjects (n = 335)	P Value [c]
Age, y	58.34 ± 9.60	58.81 ± 6.50	57.26 ± 6.90	0.86
Male	207 (53.3)	28 (52.8)	179 (53.4)	0.88
I NYHA class	77 (19.8)	11 (20.8)	66 (19.7)	0.88
II NYHA class	147 (37.9)	12 (22.6)	135 (40.3)	0.001
III NYHA class	83 (21.4)	15 (28.3)	68 (20.3)	0.001
IV NYHA class	81 (20.9)	15 (28.3)	66 (19.7)	0.001
Hypertension	214 (55.5)	31 (58.5)	183 (54.6)	0.96
Dyslipidemia	256 (66.0)	48 (90.6)	208 (62.0)	0.001
Type 2 diabetes mellitus	146 (37.6)	17 (32.1)	129 (38.5)	0.14
Obesity	172 (44.3)	14 (26.4)	158 (47.2)	0.001
Adherence to smoke	76 (19.6)	15 (28.6)	61 (18.2)	0.001
BMI (kg/m^2)	24.1 (21.6 - 28.7)	24.5 (20.4 - 25.6)	23.3 (21.5 - 24.8)	0.68
Systolic BP (mmHg)	132 ± 8	134 ± 6	131 ± 7	0.84
Diastolic BP (mmHg)	78 ± 5	79 ± 4	78 ± 5	0.92
Heart rate (beats/min)	73.45 ± 6.14	74.90 ± 4.4	72.80 ± 5.2	0.48
LVEF (%)	42.80 ± 5.76	42.31 ± 3.54	43.60 ± 4.55	0.76
GFR, 1.73 $mL/min/m^2$	82.3 (68.7 - 102.6)	81.2 (72.1 - 98.4)	83.5 (78.1 - 101.2)	0.05

[a] Abbreviations: BP, blood pressure; BMI, body mass index; GFR, glomerular filtration rate; LVEF, left ventricular ejection fraction; and NYHA, New York Heart Association.
[b] Data are presented as No. (%), mean ± SD or median and the 25% - 75% interquartile range (IQR).
[c] P value was calculated between variables for subjects who experienced the composite endpoint and those who did not.

Table 2. Biological Marker Characteristics of Study Participants [a,b]

Variables	Entire CHF Patient Cohort (n = 388)	Subjects With SH (n = 53)	Subjects Without SH (n = 335)	P Value [c]
Creatinine, μmol/L	72.3 (58.7 - 92.6)	74.2 (63.2 - 88.3)	70.5 (59.6 - 88.4)	0.068
Fasting glucose, mmol/L	5.20 (3.3 - 9.7)	5.29 (3.5 - 9.4)	5.03 (3.7 - 8.4)	0.28
HbA1c, %	6.8 (4.1 - 9.5)	6.9 (4.3 - 9.1)	6.6 (4.7 - 8.5)	0.36
Hemoglobin, g/L	135.4 (128.5 - 146.1)	134.3 (126.5 - 137.3)	137.3 (124.7 - 142.1)	0.06
Total cholesterol, mmol/L	5.2 (3.9 - 6.1)	5.4 (4.7 - 6.0)	5.0 (3.7 - 5.7)	0.047
HDL cholesterol, mmol/L	0.91 (0.89 - 1.12)	0.90 (0.85 - 1.02)	0.97 (0.92 - 1.04)	0.044
LDL cholesterol, mmol/L	3.63 (3.11 - 4.40)	3.70 (3.52 - 4.32)	3.52 (3.17 - 4.15)	0.046
Uric acid, mmol/L	33.5 (25.3 - 40.1)	36.1 (26.2 - 38.2)	30.4 (21.2 - 35.6)	0.052
NT-pro-BNP, pg/mL	1977.2 (984.7 - 2993.2)	2256.5 (995.3 - 3103.8)	1490.4 (754.5 - 2370.5)	0.036
hs-CRP, mg/L	7.34 (6.47 - 8.25)	7.95 (6.90 - 8.12)	6.92 (5.03 - 8.13)	0.036
Total T3, nmol/L	1.43 (1.09 - 2.15)	1.52 (1.12 - 2.46)	1.39 (1.11 - 2.19)	0.48
Free T3, pmol/L	5.92 (4.18 - 7.65)	5.98 (4.63 - 7.87)	5.85 (4.06 - 7.44)	0.52
Total T4, nmol/L	65.9 (68.2 - 110.3)	66.1 (68.6 - 108.5)	64.6 (67.5 - 112.7)	0.66
Free T4, nmol/L	13.5 (10.3 - 17.8)	13.8 (10.9 - 18.0)	12.9 (9.6 - 15.9)	0.48
TSH, μU/L	5.27 (2.92 - 12.1)	18.62 (11.92 - 25.4)	3.86 (2.36 - 4.57)	0.001

[a] Abbreviations: BMP, brain natriuretic peptide; EMPs, endothelial-derived apoptotic microparticles; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TSH, thyroid stimulating hormone; T4, thyroxine; and T3, triiodothyronine.
[b] Data are presented as No. (%), mean ± SD or median and the 25% - 75% interquartile range (IQR).
[c] P value was calculated between variables for subjects who experienced the composite endpoint and those who did not.

Figure 1 A demonstrates CD144+/ annexin V+ EMP numbers that were isolated from the peripheral blood of the patient cohorts. Figure 1 B demonstrates circulating CD144 +/ CD31+ EMPs in patient cohorts. Figure 1 C presents CD144+/ CD31+/annexin V+ EMP numbers that were isolated from the circulation of the patient cohorts. Figure 1 D demonstrates CD31+/annexin V+ EMP numbers that were isolated from the peripheral blood of patient cohorts. Figure 1 E demonstrates the CD62E+EMP numbers that were isolated from the peripheral blood of the patient cohorts. Figure 1 F demonstrate the CD31+/annexin V+ to CD62E+ ratio that were calculated for patient cohorts.

4.4. Correlations Between CD31+/Annexin V+ to CD62E+ Ratio and Biological Markers

We found correlations between the calculated CD31+/ annexin V+ to CD62E+ ratio and the NT-pro-BNP ($\beta = 0.59$; $r^2 = 0.192$, $P = 0.001$), TSH ($\beta = 0.52$; $r^2 = 0.178$, $P = 0.001$), hs-CRP ($\beta = 0.49$; $r^2 = 0.108$, $P = 0.001$), NYHA class ($\beta = 0.48$; $r^2 = 0.104$, $P = 0.003$), dyslipidemia ($\beta = 0.43$; $r^2 = 0.10$, $P = 0.001$), T2DM ($\beta = 0.43$; $r^2 = 0.099$, $P = 0.001$), BMI ($\beta = -0.47$; $r^2 = -0.194$, $P = 0.001$), eGFR ($\beta = -0.42$; $r^2 = -0.095$, $P = 0.001$), creatinine ($\beta = -0.38$; $r^2 = 0.150$, $P = 0.001$), SUA ($\beta = 0.36$; $r^2 = 0.093$, $P < 0.001$), male sex ($\beta = -0.29$; $r^2 = 0.071$, P

< 0.001), age (β = -0.25; r^2 = 0.055, P = 0.001) and smoking (β = -0.24; r^2 = 0.045, P = 0.001), respectively.

4.5. Associations Between CD31+/Annexin V+ to CD62E+ Ratio and Biological Markers

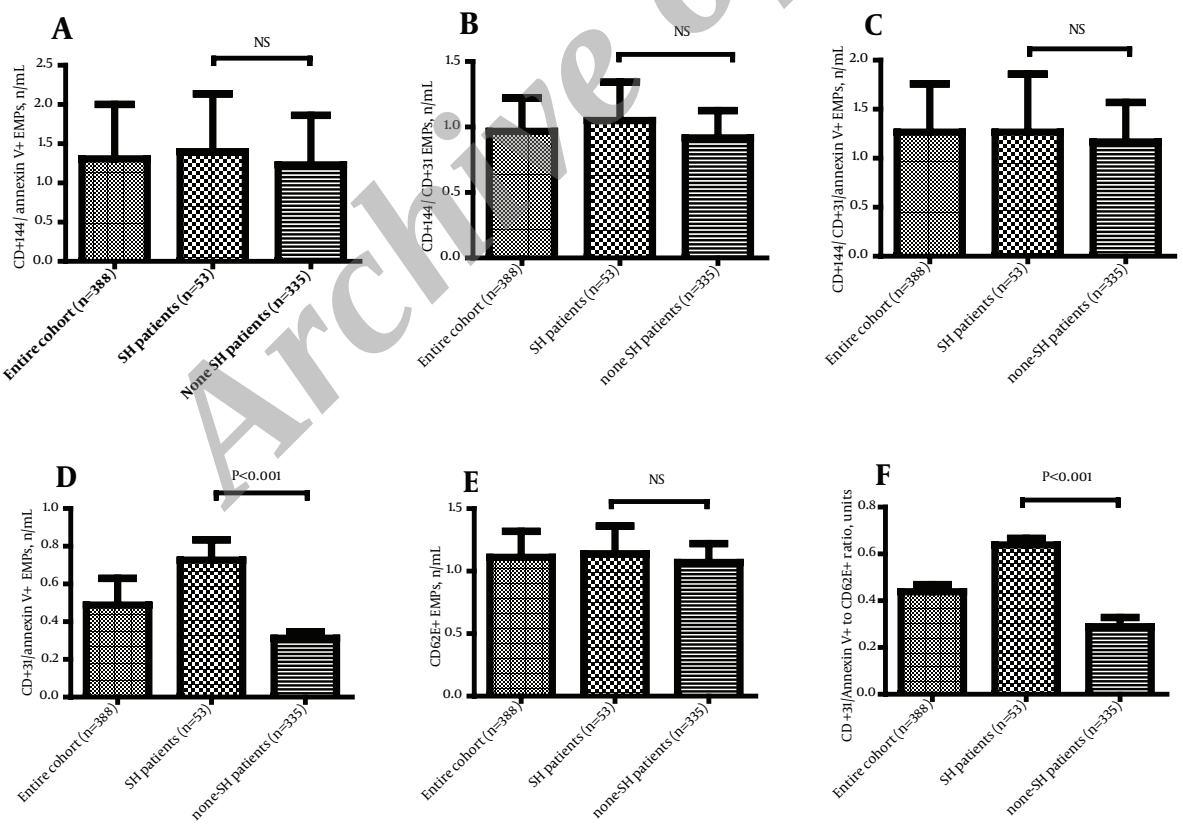
In the multivariate model, the NT-proBNP (β = 0.63), TSH (β = 0.55), NYHA class (β = 0.47), hs-CRP (β = 0.46), T2DM (β = 0.38), dyslipidemia (β = 0.36), BMI (β = -0.49), SUA (β = 0.32), and creatinine (β = -0.31) were independently associated with the CD31+/annexin V+ to CD62E+ ratio (Table 4). When NT-proBNP was eliminated from the multivariable linear regression analysis, the TSH remained independently associated with CD31+/annexin V+ to CD62E+ ratio.

Table 3. Treatment methods for the CHF patients enrolled in the study [a,b]

Variables	Entire CHF Patient Cohort (n = 388)	SH Subjects (n = 53)	None-SH Subjects (n = 335)	P Value [c]
ACE inhibitors or ARBs	388 (100)	53 (100)	335 (100)	1.0
Aspirin	305 (78.6)	42 (79.2)	263 (78.5)	0.92
Other antiplatelet drugs	83 (21.4)	11 (20.8)	72 (21.5)	0.96
Beta-adrenergic blockers	324 (83.5)	43 (81.1)	281 (83.9)	0.88
Dihydropyridine calcium channel blockers	63 (16.2)	9 (17.0)	54 (16.11)	0.88
Ivabradine	137 (35.3)	18 (34.0)	119 (35.5)	0.90
Mineralocorticoid receptor antagonists	152 (39.2)	20 (37.7)	132 (39.4)	0.76
Loop diuretics	311 (80.1)	42 (79.3)	269 (80.2)	0.83
Statins	294 (75.7)	48 (90.6)	246 (73.4)	0.012
Metformin	146 (37.6)	17 (32.1)	129 (38.5)	0.16
Sitagliptin	48 (12.4)	6 (11.3)	42 (12.5)	0.66

[a] Abbreviations: ACE, angiotensin-converting enzyme; and ARBs, angiotensin-2 receptor blockers.
[b] Data are presented as No. (%).
[c] P value was calculated between variables for subjects who experienced the composite endpoint and did not.

Figure 1. A Comparison of the Patterns of Circulating Endothelial-Derived Microparticles in Chronic Heart Failure (CHF) Patients With and Without Subclinical Hypothyroidism (SH)



Values are reported as median and IQR, and were compared using ANOVA. The line within the box represents the median value while the top and bottom lines of the box reflect the 25th and 75th percentile respectively. The top and bottom vertical lines outside of the boxes represent 10th and 90th percentile, respectively.

Table 4. Multivariable Linear Regression Analyses With the CD31+/Annexin V+ to CD62E+ Ratio as the Dependent Variable [a]

Variables	Dependent Variable: CD31+/Annexin V+ to CD62E+ Ratio	
	Standardized Coefficient β (SE)	P Value
NT-proBNP	0.63 (0.06)	< 0.001
TSH	0.55 (0.05)	< 0.001
NYHA class	0.47 (0.07)	< 0.001
hs-CRP	0.46 (0.06)	0.01
T2DM	0.38 (0.05)	0.001
Dyslipidemia	0.36 (0.06)	0.001
BMI	-0.49 (0.09)	< 0.001
eGFR	-0.30 (0.08)	0.07
Age	-0.24 (0.08)	0.7
SUA	0.32 (0.04)	0.04
Creatinine	-0.31 (0.09)	0.04
Male sex	0.25 (0.05)	0.9
Smoking	0.22 (0.05)	0.9

[a] Abbreviations: BMI, body mass index; BNP, brain natriuretic peptide; eGFR, estimated glomerular filtration rate; SE, standard error; SUA, serum uric acid; T2DM, type two diabetes mellitus; and TSH, thyroid stimulating hormone.

Table 5. Impact of Specific Factors on the Increased CD31+/Annexin V+ to CD62E+ Ratio and the Results of the Univariate and Multivariate Age- and Gender-Adjusted Regression Analysis [a]

Variances	Univariate Analysis			Multivariate Analysis		
	OR	95% CI	P Value	OR	95% CI	P Value
NT-pro-BNP, per 400 pg/mL	1.26	1.21 - 1.33	0.001	1.19	1.12 - 1.25	0.001
SH, present vs. absent	1.24	1.18 - 1.35	0.001	1.22	1.17 - 1.32	0.001
TSH, per 6.5 μU/L	1.25	1.14 - 1.42	0.001	1.23	1.13 - 1.39	0.001
T2DM, present vs. absent	1.04	1.01 - 1.07	0.001	1.03	0.99 - 1.07	0.26
NYHA class	1.12	1.06 - 1.20	0.003	1.09	1.03 - 1.14	0.001
hs-CRP, per 4.50 mg/L	1.08	1.04 - 1.13	0.001	1.05	1.03 - 1.10	0.001
Dyslipidemia, present vs. absent	1.06	1.03 - 1.12	0.002	1.06	1.04 - 1.11	0.001
BMI, per 0.5 kg/m^2	0.92	0.88 - 0.98	0.001	0.96	0.91 - 1.02	0.23
Creatinine, per 30 μmol/L	1.04	1.01 - 1.08	0.001	1.02	0.98 - 1.06	0.16
Serum uric acid, per 9.5 mmol/L	1.08	1.03 - 1.11	0.001	1.04	1.02 - 1.07	0.022

[a] Abbreviations: BNP, brain natriuretic peptide; CI, confidence interval; EMPs, endothelial-derived apoptotic microparticles; OR – odds ration; and TSH - thyroid stimulating hormone.

Using univariate and multivariate age- and gender-adjusted regression analysis, we discovered several predictor-related increases in the CD31+/annexin V+ to CD62E+ ratio in the patient study population (Table 5). The independent impact of TSH per 6.5 μU/L (odds ratio [OR] = 1.23, P = 0.001), SH (OR = 1.22, P = 0.001), NT-proBNP (OR = 1.19, P = 0.001), NYHA class (OR = 1.09, P = 0.001), hs-CRP per 4.50 mg/L (OR = 1.05, P = 0.001), dyslipidemia (OR = 1.06, P = 0.001) and serum uric acid per 9.5 mmol/L (OR = 1.04, P = 0.022) on the increase in the CD31+/annexin V+ to CD62E+ ratio, was determined.

5. Discussion

Despite the increasing evidence of the relationships between SH and the alterations in endothelial function, the effect of thyroid dysfunction on molecular mechanisms involved in endothelial injury and repair is still not completely understood. In this study, low thyroid function among CHF patients is associated with an impaired pattern of circulating EMPs with a preference for apoptotic-derived microparticles. These findings potentially reflect the association between the severity of endothelial injury and insufficiency of the reparative processes. Indeed, EMPs derived from activated endothelial cells may play a pivotal role in angiogenesis and endothelial reparation (32). Conversely, endothelial-derived microparticles originating from apoptotic endothelial cells, are considered a direct trigger of vascular injury (33, 34). It is still uncertain whether endothelial dysfunction is the result of the impaired balance between activated endothelial cell-derived and apoptotic endothelial cell-derived microparticles, respectively, or if it occurs due to disorders of vascular tone that altered the secretion of endothelial-derived microparticles (35). Considering the evidence for causality of the effects endothelial dysfunction on CV risk and CV disease progression (36), the impaired pattern of circulating EMPs may be considered as a marker of endothelial injury and dysfunction, as well as a novel CV risk factor. Diagnosis of impaired endothelial function at an early stage of CV disease and in subjects with CV risk factors is sophisticated even though the screening

of patients at high CV risk is required in order to provide preventive treatment and improve clinical outcomes (37). Elevated levels of circulating EMPs were identified in several CV risk factors (38, 39). Therefore, subclinical low thyroid function was associated with metabolic syndrome, T2DM, and insulin resistance, as well as CV disease progression (40). However, the interrelationship between SH and the impaired pattern of circulating EMPs which originated from the different origins of existing cardiac failure has not been determined. Results of our investigation demonstrate that the impaired phenotype of circulating EMPs determined as increased CD31+/annexin V+ to CD62E+ ratio, was closely associated with the NYHA class of CHF, dyslipidemia, and circulating biomarkers (hs-CRP, NT-proBNP, SUA), respectively. Although the strength of the multivariate associations between the CD31+/annexin V+ to CD62E+ ratio and the traditional biomarkers (creatinine, SUA, eGFR) were weak-to-moderate, these findings might have clinically important implications for heart failure patients. The NT-proBNP concentration and SH as well as the absolute level of TSH were identified as more sufficient factors for predicting increasing CD31+/annexin V+ to CD62E+ ratio after adjustment for common risk factors (age and gender). We believe that CD31+/annexin V+ to CD62E+ ratio was highly associated with low thyroid function, NT-proBNP concentrations, NYHA class, inflammation (hs-CRP), and kidney function biomarkers (eGFR, SUA), respectively. The CD31+/annexin V+ to CD62E+ ratio was less likely to be associated with other markers of poor outcomes and co-morbidities. However, the associations were weakened, and when NT-proBNP was added to the models, the association between hypothyroidism and CD31+/annexin V+ to CD62E+ ratio was eliminated. Thus, the imbalance between apoptotic-derived and activated endothelial cell-derived microparticles among CHF patients with SH may relate neurohumoral activation that is suitable for CHF to low thyroid function.

Although low thyroid function promotes the premature onset of subclinical atherosclerosis, it may worsen endothelial function, increase arterial stiffness and lead to disease progression among subjects with CHF (41). Therefore, dyslipidemia, low grade chronic inflammation, oxidative stress, and insulin resistance were discussed as among the main factors that contribute to CV events in SH patients (42, 43). We suggest that all these factors may affect the endothelium through changes in EMP subset presentation, which determines the interplay of several processes including inflammation, angiogenesis, adhesion, coagulation, cell survival, tissue remodeling, and tumor growth (44). In fact, EMPs incorporated in endothelial cells by their interactions with alpha4- and beta1- integrins that are expressed on the surface of microparticles. After connecting with the membranes of the target cells, EMPs exert their biological effects by directly stimulating cells or by transferring surface receptors (45). It is possible that the dysthyroid state associated with low thyroid function may worsen the expression of surface receptors, endothelial nitric oxide synthase, signal proteins, and molecules that lead to dysregulation intercellular recognition, cooperation, and information transfer (46, 47). Whether the impaired phenotype is the reason for the endothelial injury in pre-existing CHF or it appears within disease progression, remains unclear. Well planned longitudinal and randomized controlled studies on this topic are lacking, although the increased risk for CV and CHF events and mortality in SH participants are widely recognized. However, we agree with previous reports that suggest that thyroid dysfunction may considered a novel marker of CV risk and CHF progression in adult and older patient populations (7, 12, 13). Additionally, the exact understanding of the biological effects of the thyroid hormone on the ageing process, the endothelium, and cardiovascular function is still unclear and remains a clinical challenge. It remains to be determined, if the results collected from the modest sample size can be extrapolated to a larger population. High-quality studies on larger patient cohorts are needed in order to produce robust evidence of the effects of SH on the imbalance of circulating EMPs in patients with CHF and precisely recognize the molecular target for further therapy.

We suggest that the SH state in CHF patients might be associated with the impaired pattern of circulating EMPs with a predominantly increased number of apoptotic-derived microparticles.

This study has some limitations. It is necessary to note that a large pool of nanoparticles might be produced after blood sampling due to destruction of platelets and blood cells. Therefore, preparation of isolates of microparticles in samples is the most sophisticated step for further examination. Venous citrated blood was drawn from the fistula-free arm obligatorily. We believe that these risks are systemic, and in an effort to minimize them, we refused to freeze the blood samples before measuring the microparticles. Therefore, there were several technical-related difficulties in the measurement of EMPs. In fact, there is a lack of standard protocol for isolating and detecting circulating EMPs obtained from the plasma. According to the majority of experts, centrifugation became the main factor that mediated reliability of the EMP determination in samples and contributed to the biological variability of EMP count. Although the HD-FACS methodology is widely used and theoretically overlaps between two or more fluorochromes, it might reflect some obstacles to the further interpretation of obtained results. Another limitation of the present study is that a specific role for the EMPs is also possible and has not been characterized in depth in CHF patients, especially in those with various comorbidities. However, the authors believe that these restrictions may not have any significant impact on the study data interpretation. Additionally, the retrospective nature and relatively small sample size may limit the significance of the present study and increase the risk for a Type I error. The authors believe that a larger cohort of pa-

tients reporting a higher detected incidence is necessary for improving the credibility of the study.

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Authors' Contributions

Alexander E. Berezin (corresponding author) developed the hypothesis, designed the study protocol, contributed to the collection, analysis, and interpretation of the data, performed statistical analysis, and wrote the manuscript. Alexander A. Kremzer contributed to the study enrollment of the patients, collected and analyzed the data, checked for clinical events, and reviewed the source documents. Yulia V. Martovitskaya contributed to the identification of circulating biomarkers, was involved in the preparation of the microparticle isolates, in further phenotyping by flow cytofluometry, and interpreted the obtained results. Tatyana A. Samura performed the visualization procedures and analyzed the results of the examinations. Tatyana A. Berezina contributed to patient enrollment and data collection. All authors revised and edited the manuscript, finalized the draft and final version of the manuscript.

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