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**Endothelial dysfunction in subclinical hyperthyroidism:
a randomised trial**

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INTRODUCTION

Subclinical hyperthyroidism (SH) is characterised by low serum concentration of thyrotropin (TSH) in the presence of normal serum thyroid hormones and the absence of obvious symptoms of hyperthyroidism. The reported prevalence of SH is variable (depending on the area, iodine intake, inclusion of exogenous SH) and more common in women than in men (female: male ratio: 1.5: 1), and its incidence increases with advancing age.⁽¹⁾ SH is associated with increased prevalence of atrial fibrillation,⁽²⁾ and few but not all studies have shown association of SH with cardiovascular disease⁽³⁾ and increased mortality.^(2,4)

Endothelial progenitor cells have been suggested to play an important role in endothelial regeneration. Lower number of circulating endothelial progenitor cells (CEPC) in peripheral blood have been associated with increased cardiovascular risk (CVR).^(5,6) Circulating endothelial cells (CEC) have been identified as a marker of endothelial damage/injury and higher CEC levels are found in cardiovascular disorders such as peripheral vascular disease, stroke, and diabetes.⁽⁷⁾

Low levels of Nitric oxide (NO), an endothelial derived vaso-active substance, are associated with endothelial dysfunction. Asymmetric dimethylarginine (ADMA), an analogue of L-arginine, inhibit NO synthesis and therefore impair endothelial function and promote atherosclerosis. Increased ADMA levels have been reported in vascular conditions and shown as a predictor of cardiovascular disease and all-cause mortality.⁽⁸⁾

We hypothesised that SH was associated with abnormal number of CEPC, CEC or ADMA contributing to endothelial dysfunction/injury, contributing to CVR.

The primary aim of this study was to evaluate the effect of anti-thyroid drug carbimazole (compared to placebo) on CEPC and CEC by flow cytometry and ADMA in peripheral blood of SH subjects. We also evaluated Blood pressure and weight in SH after treatment with carbimazole compared to placebo.

METHODS

Thirty SH patients aged 21 to 70 years were recruited from Endocrine clinic at a tertiary hospital in Singapore after the diagnosis has been confirmed at least on 2 occasions 6 to 8 weeks apart; low serum TSH with normal thyroid hormones, both free thyroxine (FT4) and Free triiodothyronine (FT3). All study subjects had milder form of SH with detectable serum TSH due to multinodular goitre. Exclusion criteria included sick euthyroid state, recent radioiodine therapy (within 1 year of screening visit), pregnant or breastfeeding patients, and acute medical illnesses such as infections and active cancer. Due to difficulty in recruitment, we included patients with cardiovascular risk factors such as Diabetes but there is no significant difference between two groups in relation to CVR factors (Table I).

All subjects gave their written informed consent and local ethics committee approved the study. This study was registered at the clinical trials registry (ISRCTN registry with study ID ISRCTN13184358).

At the initial visit (visit 0), all subjects had anthropometric measurements (height, weight, body mass index (BMI), blood pressure, fasting bloods for CEPC, CEC, and ADMA. SH group was randomised to 2 subgroups to receive either carbimazole (5 mg) or placebo. Randomisation process was done electronically using randomization codes with the help of medical Statistician at the Clinical Research Unit in our hospital in blocks of 4 (for carbimazole or placebo pill). The study Investigators and the study subjects were blinded in the randomisation process.

At 12 weeks (visit 1), TFT was repeated and if necessary, dose of carbimazole was amended to achieve biochemical euthyroid state. Similar adjustment in the dosage (number of tablets) was done for those on placebo tablets. This dosage adjustment was done by the research co-ordinator, who was not blinded, in discussion with the principal investigator, who was blinded in the process. All subjects had similar measurements at the final visit at 6 months (18

in placebo group and 12 in carbimazole group completed the study). The recruitment period was from November 2012 to January 2018.

Serum Thyroid stimulating hormone (TSH), free thyroxine (FT4) and Free triiodothyronine (FT3) were performed on 2 Beckman Coulter DxI-800 immunoassay analysers using manufacturer-supplied reagents and calibrators. Locally derived 95% reference intervals for each of the assays were: FT4 8-21 pmol/l, FT3 3.5.-6.0 pmol/l, TSH 0.34-5.6 mU/l.

Mononuclear cells were stained with monoclonal antibodies against the following antigens: CD144 FITC (Clone 55-7H1)/ CD31 FITC (Clone WM59)/ CD133 PE (Clone W6B3C1)/ CD45 PerCP-Cy5.5 (Clone HI30)/ CD34 PE-Cy7 (Clone 8G12)/ KDR AlexaFluor 647 (Clone 89106).

Mononuclear cells were then acquired on the FACSCANTO™ flow cytometer and analysed on the FACSDiva™ software. Acquisition of 1×10^6 mononuclear cell events in total was attempted for every sample. Mononuclear cells were initially sequentially gated to exclude CD45 bright events (lymphocytes and monocytes) and to include all CD34 dim and positive events. Out of these events, circulating endothelial cells (CEC) were further defined by being CD31 bright/ CD45 negative/ CD133 negative and KDR (VEGF Receptor-2) positive, whereas endothelial progenitor cells (CEPC) are further defined by being CD31 dim/ CD45 dim/ CD133 positive and KDR positive. Suitable thresholds to define negativity were set to exclude 99.9% of negative events. CEPC and CEC levels were reported per million mononuclear cells.

Serum ADMA levels were measured by enzyme-linked immunosorbent assay (ELISA) method using a commercially available kit, following the manufacturer's instructions (Human ADMA Elisa kit, PromoKine, Heidelberg, Germany). The serum was tested without dilution. The intra-assay coefficient variation was below 20%. All samples were analysed at the end of

the study from the frozen serum samples which were stored in the freezer at -20 to 80 degree centigrade.

This was done using STATA 13. There was no previous study on CEPC, CEC, and ADMA and effect of treatment on the above markers in SH patients to calculate the sample size. We wished to recruit 40 SH patients in total but could not achieve this target due to difficult recruitment. The comparison between carbimazole and placebo were assessed using student's t-test for normally distributed data and Wilcoxon Rank Sum test for comparisons for the data which were not normally distributed, and their results were described using median and range. The results were declared as being significant if p-value is less than 0.05.

RESULTS

There was no significant difference between the two groups at baseline in relation to age, sex, BMI, blood pressure, TSH, FT4, and the presence of HT, hyperlipidaemia, Diabetes, atrial fibrillation and Ischaemic heart disease and the levels of CEPC/CEC and ADMA (Table I). There was no other significant relationship between thyroid hormones or TSH and CEPC/CEC/ADMA.

There was no significant difference in CEPC, CEC, and ADMA between Carbimazole and placebo group at 6 months (Table I). There was also no statistical difference in the above parameters if we compared the change or difference between two visits (visit 2 and visit 0 levels) in both groups. CEPC and ADMA levels reduced with Carbimazole compared to placebo but it was not statistically significant. Similarly, CEC levels rise after Carbimazole treatment but was not statistically significant.

DISCUSSION

We report for the first time that the markers for endothelial dysfunction/injury such as CEPC, CEC and ADMA are not affected in our small cohort of sub clinical hyperthyroid subjects.

The contribution of abnormal ADMA to CVR in subjects with SH has not been studied before. Few studies have evaluated ADMA levels in overt hyperthyroid state. The first study by Hermenegildo et al reported ADMA concentration in hyperthyroidism was more than twice that measured in the control group and hypothyroid group ($1.30 \pm 0.12 \mu\text{M}$ vs. 0.58 ± 0.06 and 0.57 ± 0.07 , respectively; $P < 0.01$). The L-arginine/ADMA ratio (suggesting NO availability) in patients with hyperthyroidism was 116 ± 10 , significantly lower than the values obtained in control subjects and in hypothyroid patients (190 ± 14 and 189 ± 28 , respectively; $P < 0.01$).⁽⁹⁾ Another study from China also reported higher ADMA levels in 239 subjects with hyperthyroidism due to Graves' disease.⁽¹⁰⁾ Cross-sectional data from 3689 individuals aged 20-81 years from the population-based Study of Health in Pomerania (SHIP-0) showed positive associations of ADMA with low TSH, FT3, and FT4 suggesting that the atherogenic properties of ADMA may be partially mediated by thyroid function.⁽¹¹⁾

However, we did not find any improvement in ADMA levels with carbimazole, which could be due to small number of SH subjects studied or the impact of anti-thyroid drug could take longer than 6 months. Hence, our finding needs to be confirmed in future studies assessing larger number of SH subjects.

The contribution of abnormal CEPC/CEC to CVR in subjects with SH has not been studied before. One study evaluated circulating progenitor cells in overt hyperthyroidism due to Grave's disease in small number of patients (n=23 compared to 18 controls) and reported lower number of endothelial progenitor cells in those with Graves hyperthyroidism.⁽¹²⁾ However, in that study they quantified the cultured endothelial progenitor cells in-vitro from peripheral mononuclear cells unlike in our study, in which we have quantified CEPC directly

from peripheral blood. Hence, the cells quantified in that study could be a different population of cells and subjects with subclinical hyperthyroidism was not studied in that study. In our study, after achieving euthyroid state using carbimazole in SH, there was non-significant reduction of CEPC levels (against the expected increase), thereby suggesting the contribution of CEPC to increased CVR in SH subjects could be unlikely or very minimal.

Higher number of CECs in peripheral blood has been described as a marker for damaged endothelium in several cardiovascular conditions as mentioned before.⁽⁷⁾ However, in our study, there was a non-significant increase in the level of CECs (against the expected decrease) after achieving euthyroid state. Hence, it seems unlikely that in SH, there is significant endothelial injury contributing to CVR.

The strengths of this study include evaluation of endothelial vascular markers in SH subjects using randomised placebo-controlled model and usage of stringent FACS protocol for quantifying CEPC and CEC. The disadvantage of this study includes smaller number of SH subjects mainly due to difficulty in recruitment and studying SH subjects with established vascular risk and or risk factors.

From the clinical perspective, current guidelines advocate treatment for older subjects with grade 2 SH (TSH < 0.1mU/l) due to associated morbidities such as atrial fibrillation and selected symptomatic younger SH subjects based on observational studies.^(13,14) As of now, RCTs showing cardiovascular benefit with treatment of SH is lacking. However, in our study, we could not prove existence of endothelial dysfunction/injury due to small number of SH subjects studied. To show the benefit of intervention in milder form of SH, a minimum of 40 subjects in each group would be required. The possible mechanisms of lower or impaired function of CEPCs/CECS and increased ADMA in SH include cardiac abnormalities such as ventricular dysfunction, lower nitric oxide, pro inflammatory and pro coagulant factors, and direct effect of thyroid hormones on mobilisation of CEPCs.

In conclusion, there is no evidence of endothelial dysfunction due to nitric oxide reduction or endothelial injury in our cohort of SH subjects. Due to small number of SH subjects studied, our finding needs to be confirmed in future studies with larger number of SH subjects.

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Table I: Comparisons Between SH Patients on Carbimazole And Placebo at baseline and at 6 months

Characteristics	Placebo (n=18)		Carbimazole (n=12)		p1	p2
	Visit 0	Visit 2	Visit 0	Visit 2		
Age (years)	57 (46- 63)		55 (44- 65.5)		0.96	-
Sex M/F (%)	5/13 (27.8/72.2)		3/9 (25/75)		0.99	-
Diabetes (%)	6 (33.3)		4 (33.3)		0.99	-
Hypertension (%)	8 (44.4)		5 (41.7)		0.99	-
Hyperlipidemia (%)	6 (33.3)		4 (33.3)		0.99	-
Ischemic Heart Disease (%)	1 (5.6)		1 (8.3)		0.99	-
	Visit 0	Visit 2	Visit 0	Visit 2		
BMI (kg/m ²)	23.4 (21.1- 26.3)	23.6 (21.6- 26.6)	25.0 (24.2- 26.5)	25.2 (24.3- 26.8)	0.27	0.22
Systolic BP	127.5 (119- 140)	130.0 (116-146)	128.5 (109.5-138.5)	124.5 (109.0-144.0)	0.79	0.30
Diastolic BP	71 (68-74)	70.5 (68-76)	65.5 (63.5- 82)	76.0 (68.5-82.0)	0.64	0.34
Free T4 (pmol/L)	13 (12-15)	12.5 (11-14)	13 (11.5-13.5)	11.5 (10.5-12.0)	0.45	0.06
TSH (mIU/L)	0.19 (0.14- 0.27)	0.33 (0.20- 0.48)	0.25 (0.1-0.29)	1.1 (0.7-2.3)	0.68	0.0008
CEPC (per 10 ⁶ MNC)	2.4 (1.2- 6.6)	2.0 (0.8- 4.0)	2.0 (0.3- 3.1)	1.0 (0.5- 4.2)	0.52	0.58
CEC (per 10 ⁶ MNC)	2.6 (0.0- 5.3)	3.3 (1.0- 7.0)	1.3 (0.0- 2.5)	2.1 (0.0- 3.6)	0.34	0.35
ADMA (µmol/L)	0.54 (0.49- 0.57)	0.55 (0.43-0.62)	0.56 (0.48-0.64)	0.53 (0.43-0.61)	0.47	0.7

All data except age, and sex is expressed as median and Interquartile range. p1 and p2 are the differences between placebo and carbimazole groups at visit 0 and visit 2, respectively. Abbreviations: BMI; body mass index, BP; blood pressure, TSH; thyroid stimulating hormone, FT4; free thyroxine, CEPC; circulating endothelial progenitor cells, CEC; circulating endothelial cells, ADMA; Asymmetric Di Methyl Arginine.