The Relationship of Circulating Proprotein Convertase Subtilisin/Kexin Type 9 With TSH and Lipid Profile in Newly Diagnosed Patients With Subclinical and Overt Hypothyroidism

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ABSTRACT

INTRODUCTION: Overt and subclinical hypothyroidism are mostly associated with dyslipidemia, an essential cardiovascular risk factor. Recently, thyroid stimulating hormone (TSH) was identified to have a direct role on lipid metabolism via increased expression of hepatic proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 plays a crucial role in lipid metabolism via regulating LDL-C levels. Thus, we aimed to evaluate circulating PCSK9 levels and to assess its relationship with serum TSH and lipids in newly diagnosed patients had overt and subclinical hypothyroidism.

METHODS: In our study, we enrolled 60 newly diagnosed untreated patients with overt and subclinical hypothyroidism and 30 euthyroid subjects served as the control group. Serum TSH, FT4, FT3, lipid profile and circulating PCSK9 levels using ELISA kits were measured in all subjects. Our data were summarized using mean ± SD or median and interquartile range. Correlations between PCSK9 expression levels and different variables were done using Spearman correlation coefficient.

RESULTS: Circulating PCSK9 median levels were significantly increased in patients had overt and subclinical hypothyroidism (12.45 ng/ml, 7.50 ng/ml respectively) compared to the control group (3.30 ng/ml) (P < .001). Circulating PCSK9 levels significantly correlated positively with TSH, total cholesterol, triglycerides, and BMI, and negatively correlated with FT4 and FT3 among all studied subjects. Using multivariate regression analysis TSH was the only significant independent predictor of circulating PCSK9 (P < .001).

CONCLUSION: Our results supports the new implication of TSH in lipid metabolism via the significant association with PCSK9. Whether this relationship between TSH and PCSK9 is a cause or just an association needs further evaluation.

KEYWORDS: PCSK9, TSH, lipids, subclinical hypothyroidism, overt hypothyroidism

Introduction

Thyroid hormones are crucial regulators of metabolism, development and growth. Thyroid hormones regulate different metabolic functions via the metabolism of macromolecules that affect energy homeostasis during different nutritional statuses, such as proteins, lipids and carbohydrates.1 The association between thyroid hormones and lipid metabolism has been well established.2 Overt hypothyroidism is recognized as an important risk factor for atherosclerosis and cardiovascular disease (CVD) due to its impact on lipoprotein metabolism and associated hypercholesterolemia.3 Subclinical hypothyroidism is also associated with increased serum cholesterol4 and cardiovascular disease risk.5 Thyroid hormones can affect lipid metabolism by various mechanisms by inducing cholesterol synthesis and enhancing the clearance of low density lipoprotein cholesterol (LDL-C) and the metabolism of high density lipoprotein cholesterol (HDL-C).6-8 Recently, a direct role of thyroid stimulating hormone (TSH) in lipid metabolism was identified via increased hepatic expression of proprotein convertase subtilisin/kexin type 9 (PCSK9).9

PCSK9 represents the ninth member of substilisin family of kexin-like pro-convertases which was previously known as neural apoptosis regulated convertase 1 (NARC-1) and is involved in the breakdown of different precursor proteins such as neuropeptides, prohormones, cytokines, growth factors and various cell surface proteins.10 PCSK9 is mainly expressed and secreted by hepatocytes; however, it is also expressed in other sites such as the intestine, kidney, lung, brain, endothelial, and smooth muscle cells of blood vessels.11-13

PCSK9 is crucial for regulating circulating LDL-C through degradation of LDLRs (low density lipoprotein receptors), thus; reducing LDLR expression on the hepatic cell surface, which prevents the clearance of LDL-C resulting in elevation in circulating LDL-C levels.14 LDL-C is
regulated by the coordinated interaction of LDLR and PCSK9 which is under the control of intracellular cholesterol levels and SREBP-2 (transcription factor sterol regulatory element binding protein-2). A decrease in the intracellular cholesterol levels stimulates SREBP-2 to induce LDLR gene expression increasing LDLRs and promoting the removal of LDL-C from the circulation. Simultaneously, SREBP-2 enhances the expression of PCSK9 which induces LDLR degradation. In addition, PCSK9 regulates other receptors and lipid proteins such as very low density lipoprotein receptor (VLDLR), LDLR related protein1 (LRP1), and lipoprotein E receptor.

Increased PCSK9 gene expression due to gain of function mutation was identified in familial hypercholesterolemia and in individuals with elevated serum LDL-C levels, which was associated with increased cardiovascular risk factors. Additionally, decreased PCSK9 gene expression due to loss of function mutations was associated with low LDL-C levels and decreased cardiovascular risk. Furthermore, different PCSK9 expression levels were reported in diabetes mellitus, nonalcoholic fatty liver, dyslipidemia and atherosclerotic risk disorders. In our study, we aimed to assess circulating PCSK9 expression levels in patients with overt and subclinical hypothyroidism and to assess the relationship of circulating PCSK9 expression levels with TSH and serum lipids in this group of patients.

Subjects and Methods

Subjects

We enrolled 90 subjects in our study, and calculated our sample size relying on literature effect size of 1.4986 in PCSK9 levels as a primary outcome that was a large effect size allowed us to calculate our sample size at a higherpower level of 95% instead of 80%, 30 patients were required to achieve 95% power at 5% level of significance. Our sample size calculation was performed using G’Power software version 3.1.9.4 (Germany). Studied subjects were divided into 3 groups: 30 patients had subclinical hypothyroidism and 30 patients had overt hypothyroidism, and 30 apparently healthy subjects served as the control group age and sex matched. Patients were recruited from the outpatient clinic of endocrinology, Kasr Al Ainy Hospital, Cairo University, from May 2021 to July 2021. Enrolled patients were newly diagnosed untreated for thyroid dysfunction. Patients with subclinical hypothyroidism included 3 males and 27 females with a mean age of 36 years, while patients with hypothyroidism included 8 males and 22 females with a mean age of 38 years. Control subjects included 6 males and 24 females with a mean age of 40 years. Subclinical hypothyroidism diagnosed by elevated serum TSH levels was associated with normal thyroid hormone levels, while overt hypothyroidism was diagnosed by elevated serum TSH levels associated with low thyroid hormone levels. We excluded patients with positive anti-thyroid peroxidase antibodies or patients on thyroxin treatment for thyroid dysfunction or taking lipid lowering drugs. Additionally, we excluded patients who had diabetes mellitus, chronic liver or kidney diseases, ischemic heart disease, chronic autoimmune disorders, malignancy, pregnancy or smokers.

Ethical aspects: The study was performed in agreement with the Helsinki ethical declaration guidelines 1975. We obtained an informed voluntary written consent from all enrolled subjects. Our study was approved by the Internal Medicine Research Ethical Committee department, Cairo University, approval number (136-2021).

Methods

All patients were subjected to thorough history taking and full clinical examination. Body mass index (BMI) was calculated in every subject by dividing the body weight in kilograms (kg) by square height in meters (m²). Three milliliters of venous fasting blood samples were collected from every subject for laboratory investigations. The collected venous blood samples were left to clot at room temperature before centrifugation at 1000 × g for 15 minutes. The formed supernatant was collected and put in sterile tubes and stored at −80°C until needed. Fasting blood sugar (FBS), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TGs), thyroid stimulating hormone (TSH), free thyroxine (FT4), free triiodothyronine (FT3), and proprotein convertase subtilisin/kexin type 9 (PCSK9) were measured in all subjects.

Serum TSH was measured by a DRG TSH ELIZA (solid phase enzyme-linked immunosorbent assay) kit that was ranging from 0.4 to 5 µIU/ml was considered normal. Serum FT3 was measured by DRG FT3 (EIA-3801) (DRG International Inc., USA) a solid phase competitive enzyme immunoassay. FT3 (ranging from 1.4-4.2 pg/ml) was considered normal. Serum FT4 was measured by DRG FT4 (DRG International Inc., USA); FT4 (ranging from 0.8 to 1.9 ng/ml) was considered normal. TC was measured using established enzymatic methods using a Stanbio kit (USA). HDL-C was assessed by the HDL-C precipitant method. TGs were measured enzymatically. LDL-C concentrations were calculated by Friedewald’s formula. Serum PCSK9 was estimated using ELISA kits supplied by Elbascience.
addition of a stop solution to each microplate, the enzyme substrate reaction stopped, and the color changed into yellow. The optical density (OD) was measured at a wavelength of 450 nm, which is proportional to the amount of human PCSK9. The concentration of human PCSK9 in the samples was calculated by comparing the OD of the samples to a standard curve.

Statistical methods

Our data were coded and entered using the statistical package for the Social Sciences (SPSS) version 26 (IBM Corp., Armonk, NY, USA). Means and standard deviations were applied for normally distributed quantitative variables, and medians and interquartile ranges were used for non-normally distributed quantitative variables. For categorical variables, frequencies (number of cases) and relative frequencies (percentages) were used. Our groups were compared using analysis of variance (ANOVA) associated with multiple comparisons using post hoc test in normally distributed quantitative variables and nonparametric Kruskal-Wallis test and Mann–Whitney test for non-normally distributed quantitative variables. For comparing categorical data, the chi square ($\chi^2$) test was performed. An exact test was used instead when the expected frequency was less than 5. Correlations between quantitative variables were performed using Spearman correlation coefficient. Linear regression analysis was performed to determine whether the relationship between PCSK9 and TSH still significant after adjustment of possible confounders as TC and TGs. Also multivariate stepwise regression was performed using PCSK9 as dependent variable and TSH, BMI, FT3, FT4, TC and TGs as independent predictors. $P$ values less than .05 were considered statistically significant.

Results

Our demographic and laboratory data are shown in Table 1. The subclinical hypothyroid patients included 27 (80%) females and 3 (10%) males with mean age (36.03 ± 13.6 years), the overt hypothyroid group included 22 (73%) females and 8 (26.7%) males with mean age (38.17 ± 9.75 years) and the control group included 24 (80%) females and 6 (20%) males with mean age (40.4 ± 13.64 years) with nonsignificant difference regarding age ($P$ = .40) and sex ($P$ = .252) between groups (Table 1).

| VARIABLES       | CONTROL GROUP | SUBCLINICAL HYPOTHYROIDISM | OVERT HYPOTHYROIDISM | P VALUE |
|-----------------|---------------|---------------------------|----------------------|---------|
|                 | COUNT %       | COUNT %                   | COUNT %              |         |
| Sex             |               |                           |                      |         |
| Male            | 6 20.0        | 3 10.0                    | 8 26.7               | .252    |
| Female          | 24 80.0       | 27 90.0                   | 22 73.3              |         |
| Age (years)     | 40.40 ± 13.64 | 36.03 ± 13.63             | 38.17 ± 9.75         | .403    |
| Weight (kg)     | 72.02 ± 17.62 | 78.14 ± 24.53             | 82.15 ± 13.78        | .126    |
| Height (cm)     | 160.22 ± 9.47 | 161.20 ± 6.90             | 162.73 ± 6.72        | .455    |
| BMI (kg/m²)     | 27.91 ± 5.85  | 30.00 ± 9.07              | 31.02 ± 5.04         | .207    |
| TC (mg/dl)      | 202.90 ± 34.92| 196.13 ± 35.80            | 227.33 ± 39.46       | .004*   |
| TGs (mg/dl)     | 116.40 ± 38.58| 137.40 ± 43.01            | 161.37 ± 61.22       | .002*   |
| HDL-C (mg/dl)   | 37.47 ± 11.99 | 39.60 ± 9.00              | 41.40 ± 10.37        | .554    |
| LDL-C (mg/dl)   | 125.47 ± 21.72| 117.23 ± 29.94            | 124.03 ± 36.36       | .526    |
| FBS (mg/dl)     | 93.93 ± 9.66  | 89.23 ± 9.46              | 92.87 ± 9.23         | .136    |

Data are presented as mean ± SD; *$P$ < .05 was considered statistically significant.

Abbreviations: BMI, body mass index; TC, total cholesterol; TGs, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; FBS, fasting blood sugar.

Table 1. Demographic and laboratory data of the studied groups.
Table 2. Comparison of serum FT3, FT4, TSH and pCSK9 levels in the studied groups.

|                      | CONTROL GROUP | SUBCLINICAL HYPOTHYROIDISM | HYPOTHYROIDISM |
|----------------------|---------------|----------------------------|----------------|
| FT3 (pg/ml)          | 3.15          | 2.80                       | 3.61           |
| FT4 (ng/ml)          | 1.30          | 3.05                       | 1.30           |
| TSH (µIU/ml)         | 1.55          | 8.14                       | 5.87           |
| pCSK9 (ng/ml)        | 3.30          | 7.50                       | 7.50           |

P VALUE

|                      | 1ST QUARTILE  | 3RD QUARTILE  | 1ST QUARTILE  | 3RD QUARTILE  |
|----------------------|---------------|---------------|---------------|---------------|
| FT3 (pg/ml)          | 2.80          | 3.61          | 2.75          | 3.15          |
| FT4 (ng/ml)          | 1.10          | 0.96          | 0.95          | 1.30          |
| TSH (µIU/ml)         | 1.05          | 3.11          | 0.67          | 1.55          |
| pCSK9 (ng/ml)        | 0.67          | 8.14          | 3.30          | 1.55          |

Data are presented as median and interquartile range; *p < .05 was considered statistically significant.

Comparison of the circulating PCSK9 levels among the studied groups showed significant difference in the median levels between the 3 groups with (P<.001) which associated with significant elevation in the overt hypothyroid group (12.45 ng/ml) and the subclinical hypothyroid group (7.50 ng/ml) compared to the control group (3.30 ng/ml) (Table 2, Figure 1) and showed significant difference by comparing each 2 groups separately (P<.001).

Using Spearman correlation to detect the association of the serum PCSK9 levels with different parameters among all added subjects (n=90), we found a significant positive correlation of serum PCSK9 with weight (r=0.276, P=.008), BMI (r=0.210, P=.047), TC (r=0.211, P=.046), and TGs (r=.352, P=.001) (Table 3, Figure 2). Regarding the association between serum PCSK9 levels and thyroid functions, we found a positive correlation with TSH (r=0.753, P=.001) and negative correlation with FT4 (r=-0.682, P<.001) and FT3 (r=-0.462, P<.001) (Table 3, Figure 3). Also we found significant correlation between serum TSH with BMI (r=0.239, P=.024), TC (r=0.265, P=.012), TGs (r=0.403, P<.001) (Table 3, Figure 4) and significant negative correlation with FT4 (r=-0.736, P<.001) and FT3 (r=-0.489, P<.001) among all subjects (Table 3). Using multivariate linear regression analysis for adjustment of BMI, TC and TGs as possible confounders we found that TSH was still significantly correlated with the circulating PCSK9 (β=0.059, P<.001), r²=0.296 (Table 4). Further analysis by stepwise regression using PCSK9 as the dependent variable and TSH, BMI, FT3, FT4, TC, and TGs as co-variables, revealed that TSH was the only independent predictor of PCSK9 ( β=0.067, P<.001), r²=0.278 (Table 5).

Discussion

The crucial regulatory role of thyroid hormones on all aspects of lipid metabolism is of great importance due to its association with cardiovascular risk factors. Overt and subclinical hypothyroidism is associated with dyslipidemia especially elevation in cholesterol and LDL-C levels which are significantly normalized with thyroxine treatment. Various studies have showed a significant association between increased TSH levels and lipids, which has also been documented even with normal TSH levels.

In our study we found increased frequency of females compared to males in the studied subjects. Other studies documented increase prevalence of thyroid dysfunction in females compared to males which was linked to the direct effect of estrogen on thyroidal cells proliferation and function. Also we found a significant increase in total cholesterol and triglycerides in patients with subclinical and overt hypothyroidism compared to healthy subjects. Additionally, there was a significant linear association between TSH levels and total
cholesterol, triglycerides and BMI in the studied groups. Different mechanisms of thyroid hormones on lipid metabolism have been described. Thyroid hormones can stimulate cholesterol synthesis by inducing LDLR gene transcription and hydroxymethylglutaryl coenzyme A reductase (HMGCR). Simultaneously, thyroid hormones also induce SREBP-2, which increases LDLR expression, leading to clearance of LDL-C. Thus, in hypothyroidism, there is a decrease in SREBP-2 resulting in a decrease in LDLRs, which causes a decrease in LDL-C clearance and hypercholesterolemia. In addition, thyroid hormones induce cholesterol ester transferase activity, which increases the metabolism of HDL-C. Additionally, TSH can reduce hepatic bile acid synthesis via the SREBP2 signaling pathway independent of thyroid hormones. Thus, the National Cholesterol Education Program recommended screening subjects with dyslipidemia for thyroid dysfunction before statin treatment. Moreover, current evidence suggests the introduction of lipid-lowering medications

Figure 1. Box plot graph shows the comparison of serum PCSK9 (A), TSH (B), FT4 (C), FT3 (D) between the studied groups.
Clinical Medicine Insights: Endocrinology and Diabetes

in patients with hyperlipidemia and subclinical hypothyroidism regardless of the decision for thyroxin treatment.46

Recently, TSH was shown to have a direct effect on dyslipidemia via elevation of PCSK9 expression in HepG2 cells through the stimulation of SREBP1c and SREBP2.9 Therefore, we investigated the relationship between circulating PCSK9 levels and TSH and lipids in newly diagnosed untreated patients with subclinical and overt hypothyroidism, and we found a significant elevation in circulating PCSK9 levels in patients with subclinical and overt hypothyroidism compared to the control group. Additionally, we found that circulating PCSK9 correlated positively with serum TSH and negatively with FT4 and FT3. Our findings were in line with 2 other studies that showed significant increase in circulating PCSK9 levels in patients with short-term subclinical hypothyroidism,

Figure 2. Scatter plot graph demonstrates the positive correlation between circulating PCSK9 with BMI (A), TG (B), and TC (C) among the studied subjects.

Table 3. Correlation of PCSK9 and TSH with different parameters in the added studied groups (n=90).

| BMI | TC | TG | HDL-C | LDL-C | TSH | FT3 | FT4 | FBS |
|-----|----|----|-------|-------|------|-----|-----|-----|
| r   | 0.210 | 0.207 | 0.352 | 0.328 | 0.239 | 0.265 | 0.169 | 0.135 |
| P   | 0.047 | 0.047 | 0.001 | 0.001 | 0.024 | 0.012 | 0.012 | 0.012 |

r = Spearman correlation co-efficient, *P < 0.05 is significant.
and overt hypothyroidism was significantly associated with elevations in total cholesterol, LDL-C and TSH and negatively correlated with FT4 in patients with subclinical hypothyroidism.\textsuperscript{27,47} Other studies on euthyroid individuals also documented significant positive correlations between PCSK9 and TSH and lipids and negative correlations with FT3 and FT4.\textsuperscript{48,49} In contrast, Gagnon et al did not find a significant relation between PCSK9 levels and TSH after acute administration of rhTSH in euthyroid subjects suggesting that chronic TSH stimulation is needed to affect hepatocyte PCSK9 secretion and elevation in human serum.\textsuperscript{50}

We further analyzed the relation between circulating PCSK9 and lipids and found a significant association between PCSK9 and total cholesterol but we did not find significant association between PCSK9 and LDL-C. Various studies documented the significant relation between PCSK9 and hypercholesterolemia due to the regulatory effect of PCSK9 on LDL-C.\textsuperscript{51,52} The nonsignificant association of PCSK9 with LDL-C in our study was consistent with other studies.\textsuperscript{53,54} It was estimated that variation in circulating PCSK9 was only responsible for 7% of the variation in serum LDL-C, suggesting other metabolic and genetic determinants.\textsuperscript{55}

The relationship of PCSK9 expression and triglyceride metabolism is still unclear. In our study, we found a significant positive association between circulating PCSK9 and triglycerides. Similarly, in the Dallas Heart Study, a significant association of PCSK9 levels with serum triglycerides was reported.\textsuperscript{56} Additionally, the study by Kwakernaak et al reported a positive correlation of PCSK9 levels with triglycerides, which was linked to the direct effect of PCSK9 on the intermediate low density lipoprotein (IDL), a triglyceride-rich lipoprotein.\textsuperscript{48} In addition, increased expression of PCSK9 was found to be associated with increased production of intestinal triglyceride rich apolipoprotein B via the LDLR pathway and independently through microsomal transfer protein (MTP).\textsuperscript{57}

We also found a significant linear association between PCSK9 and BMI among the studied subjects which was also

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Scatter plot graph demonstrates the correlation between circulating PCSK9 with TSH (A), FT4 (B), and FT3 (C) among the studied subjects.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Scatter plot graph demonstrates the correlation between TSH with BMI (A), TC (B) and TG (C) among the studied subjects.}
\end{figure}
Recently, PCSK9 was found to be expressed in human visceral adipose tissue, and its expression levels were correlated with BMI. In contrast, other studies did not find a significant association of PCSK9 with BMI. Due to the crucial role of PCSK9 in lipid metabolism and metabolic parameters, clinical studies documented a reduction in LDL-C levels via PCSK9 inhibitors in healthy subjects and in patients with hyperlipidemia and associated cardiovascular risk. Using stepwise regression analysis, we found that TSH was only the significant independent predictor of PCSK9 which accounts 28% of variation. In the study by Susan et al reported different significant predictors in normal population to circulating PCSK9 levels as age, gender and ethnicity, metabolic and genetic variations that only account 23% of variation. These results highlights the presence of other contributing factors influencing the serum PCSK9 levels that still undetermined and need further research.

Limitations of our study presented in the relatively small number of patients studied. In addition, our study was a cross-sectional study, it would have been great if we assessed the correlation of circulating PCSK9 levels with other variables in a longitudinal study. Besides, assessment of PCSK9 after normalization of thyroid functions is important. Also, enrollment of specific gender either males or females is better as there were contradictory results about gender significance regarding PCSK9 levels which needs further research.

In conclusion, our results showed significant elevation in circulating PCSK9 levels in newly diagnosed patients with thyroid dysfunction. PCSK9 was positively associated with TSH levels, BMI, TC and TGs and negatively associated with FT3 and FT4 levels. TSH was only the significant independent predictor of PCSK9 which accounts 28% of variation. Our results support the new implication of TSH in lipid metabolism via the significant association with PCSK9. Whether this relationship between TSH and PCSK9 is a cause or just an association needs further evaluation. Other contributing factors affecting circulating PCSK9 levels in patients with thyroid dysfunction need to be evaluated.

**Author Contribution(s)**
NA: study design, data analysis, writing the original manuscript, reviewing, editing and approved the final manuscript; LR: Methodology, reviewing, and approved the final manuscript; SES: Data collection and analysis, reviewing and approved the final manuscript.

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