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Pre-β-HDL formation relates to high-normal free thyroxine in type 2 diabetes mellitus

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ABSTRACT

Objectives: Low-normal thyroid function within the euthyroid range may influence plasma lipoprotein levels. Associations between variation in thyroid function and pre-β-high density lipoproteins (pre-β-HDL), i.e. lipid-poor or lipid free HDL particles that act as initial acceptor of cell-derived cholesterol, are unknown. We determined relationships of plasma pre-β-HDL with thyroid function in euthyroid subjects with and without type 2 diabetes mellitus (T2DM).

Design and Subjects: TSH, free T4, plasma (apo)lipoproteins, pre-β-HDL, pre-β-HDL formation (pre-β-HDL generation during incubation with lecithin:cholesterol acyltransferase being inhibited) and phospholipid transfer protein (PLTP) activity were measured in fasting plasma from 72 T2DM and 82 non-diabetic subjects.

Results: TSH was similar and free T4 was slightly higher ($P < 0.05$) in T2DM vs. non-diabetic subjects. HDL cholesterol and apoA-I were lower, whereas pre-β-HDL (expressed as % of apoA-I), triglycerides and PLTP activity were higher in T2DM ($P < 0.05$ to $P < 0.001$). In T2DM, pre-β-HDL formation (in apoA-I concentration and in % of apoA-I) was positively related to free T4, PLTP activity, total cholesterol and triglycerides ($P < 0.05$ for each). Multivariable linear regression analyses, adjusted for age, sex, PLTP activity, total cholesterol and triglycerides, demonstrated that pre-β-HDL formation was positively related to free T4 (in apoA-I concentration: $\beta = 0.278$, $P = 0.014$; in % of apoA-I: $\beta = 0.343$, $P = 0.003$) in T2DM, but not in non-diabetic subjects (both $P > 0.30$; interaction terms: both $P < 0.05$).

Conclusions: Variations in thyroid function within the euthyroid range may influence the metabolism of pre-β-HDL in T2DM.

1. Introduction

Low-normal thyroid function, as inferred from higher TSH or lower thyroid hormone levels within the euthyroid range, may confer changes in plasma lipids and other biomarkers that relate to increased cardiovascular risk [1,2]. Single determinations of TSH and free T4 can provide relevant information regarding the effect of thyroid function status on plasma lipids and lipoproteins [2,3]. In agreement with this concept, low-normal thyroid function associates with a greater carotid intima media thickness (cIMT), an established marker of subclinical atherosclerosis [4,5]. Higher TSH levels within the euthyroid range may also predict cardiovascular mortality in women [6].

It is likely that low-normal thyroid function predicts higher plasma levels of plasma total cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides, but associations with high density lipoprotein (HDL) cholesterol have been inconsistently reported [2]. HDL particles are very heterogeneous in size, structure and composition with important consequences for their functional properties [7,8]. This underscores the relevance to discern the relationship of HDL subfractions with variations in thyroid function in more detail. In this regard, it is important that a small proportion of HDL consists of lipid poor or lipid free particles, designated pre-β-HDL [9,10]. By promoting cellular cholesterol efflux, pre-β-HDL particles play an important role in the reverse cholesterol transport pathway, whereby cholesterol is transported from peripheral cells back to the liver for biliary transport and excretion in the feces [8,9–11]. Although not unequivocally reported [12], higher plasma pre-β-HDL concentrations are observed in subjects with cardiovascular disease [13,14]. Of further relevance, higher plasma pre-β-HDL levels associate with a greater cIMT, both in diabetic and non-diabetic subjects [15,16]. It is plausible to interpret such higher pre-β-HDL levels in the context of increased cardiovascular risk to be indicative of impaired conversion of pre-β-HDL to more mature cholesterol-rich HDL particles, and hence to reflect impaired HDL-mediated reverse cholesterol transport [17].

Relationships of several HDL-mediated functional properties such as the cholesteryl ester transfer protein (CETP)-mediated transport of cholesteryl esters out of HDL, as well as an impaired ability of HDL to protect oxidative modification of LDL in vitro were recently reported to be particularly evident in individuals with Type 2 diabetes mellitus (T2DM) [18,19]. In view of the greater cIMT in conjunction with higher

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pre-β-HDL levels in T2DM [15], it is relevant to assess whether the hitherto unexplored association of pre-β-HDL lipoprotein with thyroid function varies according to diabetes status.

The present study was initiated to discern in subjects with and without T2DM whether plasma pre-β-HDL is associated with variations in thyroid function within the euthyroid range. Second, we determined the extent to which such relationships are modified in the context of T2DM.

2. Subjects and methods.

2.1. Patients and methods

The study was performed in a University Hospital setting, and was approved by the medical ethics committee of the University Medical Center **Groningen, The Netherlands.** Caucasian participants (aged >18 years) were recruited by advertisement, and had provided written informed consent. T2DM had been previously diagnosed by primary care physicians using guidelines from the Dutch College of General Practitioners (fasting plasma glucose ≥7.0 mmol/l and/or non-fasting plasma glucose ≥11.1 mmol/l). T2DM patients who were treated with metformin and/or sulfonylurea were allowed to participate, but patients using other glucose lowering drugs and/or insulin were excluded. The use of anti-hypertensive medication was allowed. Eligible subjects had a serum TSH and a free T4 level within the institutional reference range (see below). Additional exclusion criteria were clinically manifest cardiovascular disease, renal insufficiency (estimated glomerular filtration rate < 60 ml/min/1.73 m^2 and ⁄ or urinary albumin >20 mg/l), liver disease (serum transaminase levels >2 times above the upper reference limit), pregnancy and use of lipid lowering drugs. Subjects who used other medications (except for oral contraceptives), current smokers and subjects who used >3 alcoholic drinks daily were also excluded. Physical examination did not reveal pulmonary or cardiac abnormalities. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Blood pressure was measured after 15 min of rest at the left arm using a sphygmomanometer. The participants were evaluated between 08.00 and 10.00 h after an overnight fast.

2.1.1. Laboratory measurements.

Serum and EDTA-anticoagulated plasma samples were stored at −80 °C until analysis. Plasma glucose and glycated hemoglobin (HbA1c) levels were measured shortly after blood collection.

Serum TSH (sandwich principle; Roche Diagnostics GmbH., Mannheim, Germany, cat. no. 117,314,591; reference range 0.5–4.0 mU/l) and free T4 (competition principle; Roche Diagnostics GmbH., Mannheim Germany, cat. no. 11,731,297; reference range 11.0–19.5 pmol/l) were measured by electrochemiluminescence immunoassay using a Modular Analytics immunoassay analyzer. The inter-assay coefficients of variation (CVs) were <5%.

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11,876,023 and 11,875,540, respectively; Roche Diagnostics GmBH, Mannheim, Germany). HDL cholesterol was measured with a homogeneous enzymatic colorimetric test (Roche/Hitachi, cat no 04,713,214; Roche Diagnostics GmbH, Mannheim, Germany). LDL cholesterol was calculated by the Friedewald formula in case of plasma triglycerides <4.5 mmol/l. ApoA-I was assayed by immunoturbidimetry (Roche/Cobas Integra Tina-quant catalog no. 03,032,566, Roche Diagnostics GmBH, Mannheim, Germany).

Plasma pre-ß-HDL was measured by crossed immuno-electrophoresis as described [16,20]. In brief, plasma samples were thawed while kept on ice. 0.9 μmol/l Pefabloc SC (Boehringer-Roche, Penzberg, Germany) and 1.8 μg/l Trasylol (Bayer, Mijdrecht, The Netherlands) were added to inhibit proteolysis (both final concentrations). The crossed immuno-electrophoresis consisted of agarose electrophoresis in the first dimension for separation of lipoproteins with pre-β- and α-mobility. Antigen migration from the first agarose gel into the second agarose gel, containing goat anti-human apo A-I antiserum, was used to quantitatively precipitate apo A-I. The antiserum was monospecific for human apo A-I using an immunodiffusion assay. Lipoprotein electrophoresis was carried out in 1% (weight/vol) agarose gels in Tris (80 mmol/l)-tricine (24 mmol/l) buffer, 5% (vol/vol) polyethylene glycol 300 (pH 8.6) and run in an LKB 2117 system (4 °C for 3 h, 210 V). An amount of 3 μl plasma was added to each well. The track of the first agarose gel was excised and annealed with melted agarose to a gel containing 0.66% (*v*/*v*) goat anti-human apo A-I anti-serum (Midland Bioproducts corporation, Boone Iowa) and 0.01% Tween 20 (*w*/*v*), that was cast on GelBond film (Amersham, Uppsala, Sweden). The plate was run in an LKB 2117 system (4 °C for 20 h, 50 V) in Tris-tricine buffer. Unreacted antibody was removed by extensive washing with saline. The gel was stained with Coomassie Brilliant Blue R250, dried, and scanned with a HP scanjet 5470c. Areas under the pre-β-HDL and α-HDL peaks were calculated. The preß-HDL area was expressed as the percentage of the sum of apo A-I in the pre-β-HDL and the α-HDL areas. Plasma pre-ß-HDL formation, i.e. the ability of plasma to generate pre-ß-HDL, was determined using the same procedure but now after 24 h incubation of plasma at 37 °C under conditions of lecithin:cholesterol acyltransferase (LCAT) inhibition [20]. To this end iodoacetate (final concentration 1.0 mmol/l) was added directly after thawing the plasma samples. Pre-ß-HDL and pre-ß-HDL formation were calculated using the total plasma apo A-I concentration (expressed in apoAI (g/l), and alternatively in % of total plasma apoA-I. The inter-assay CVs were <9%.

Plasma PLTP activity was assayed with a phospholipid vesicles-HDL system, using [^{14}C]-labeled dipalmitoyl phosphatidylcholine as described [20]. Briefly, plasma samples (1 μl) were incubated with [^{14}C]-phosphatidylcholine-labeled phosphatidylcholine vesicles and excess pooled normal HDL for 45 min at 37 °C. The method is specific for PLTP activity. Plasma PLTP activity levels vary linearly with the amount of plasma added to the incubation system. PLTP activity was related to the activity in human reference pool plasma and was expressed in arbitrary units (AU; 100 AU corresponds to 13.6 μmol phosphatidylcholine transferred per mL per h). The inter-assay CV of PLTP activity was 5%.

2.1.2. Statistical analysis

SPSS version 22.0 was used for data analysis. Data are expressed as means ± SD, medians (interquartile ranges) or in numbers. Differences between subjects with and without T2DM were determined by unpaired *t*-tests or Chi-square tests where appropriate. Plasma triglycerides were not parametrically distributed, and were logarithmically transformed for analysis. Univariate relationships were calculated using Pearson correlation coefficients.

Multivariable linear regression analyses were performed to disclose the independent relationships of plasma pre-β-HDL formation and (apo)lipoproteins with thyroid function parameters. In addition, multivariable linear regression analyses were carried out to determine interactions of diabetes status with thyroid function parameters impacting on pre-β-HDL formation. Interaction terms were calculated as the product terms of TSH with the presence of T2DM or with HbA1c. To account for possible outliers the distributions of continuous variables were centered to their mean value by subtracting the individual value from the group mean value. Interaction terms were considered to be statistically significant at two-sided *P*-values <0.10 [21]. Otherwise, the level of significance was set at two-sided *P*-values <0.05.

3. Results

Of 170 potentially eligible subjects, 16 subjects were excluded based on either a TSH or a free T4 level outside the reference range. As a result, 72 T2DM subjects and 82 non-diabetic control subjects participated in the study (Table 1). In T2DM subjects, median diabetes duration was 5.4 years. All T2DM subjects had been given dietary advice. Nineteen T2DM patients used metformin and 19 patients used sulfonylurea alone. Both drugs were used by 24 patients. Other glucose lowering