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Original Research Article

Overt hypothyroidism is associated with blood inflammatory biomarkers dependent of lipid profile

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ARTICLE INFO

Article history:
Received 10 June 2015
Received in revised form
6 October 2015
Accepted 8 October 2015
Available online 21 October 2015

Keywords:
Hypothyroidism
Lipids
Cytokines
Inflammation
Cell-free DNA

ABSTRACT

To investigate the association between inflammatory biomarkers and overt hypothyroidism (OH). We measured inflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) as well as cell-free DNA (cf-DNA) levels in 40 OH patients and 40 healthy controls. Total cholesterol, high and low density lipoprotein subfractions, triglyceride, fibrinogen, and D-dimer were recorded. Increased inflammatory profile was evidenced through significant elevations in the concentrations of all cytokines and cf-DNA levels in the OH group. Lipids and prothrombotic markers were also increased in hypothyroid subjects. A significant association between the inflammatory cytokines and lipid profile was observed. A multivariate analysis showed that this result was independent of the sex, age and BMI status of the subjects. Hypothyroidism is associated with proinflammatory state. Lipid abnormalities have a stronger influence on inflammation, increasing cardiovascular risk and atherosclerosis development in hypothyroidism.

Introduction

Overt hypothyroidism (OH) is characterized by high thyroid-stimulating hormone (TSH) blood concentration, low triiodothyronine (T3) and thyroxine levels (T4), and alterations of plasma lipid concentration, which could also be involved in the progress of atherosclerosis (Donnini et al., 2003). Hypercholesterolemia is very common in OH patients, mainly due to higher low-density lipoprotein cholesterol

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1214-021X/$ – see front matter
http://dx.doi.org/10.1016/j.jab.2015.10.002

(LDL-C) levels. In addition, OH presents association with other atherosclerosis risk factors such as diastolic hypertension, coagulopathy and impaired endothelial function. As described in the review performed by Ichiki (2010) who summarized the basic and clinical studies on the role of thyroid hormone in atherogenesis, emerging risk factors have been associated with atherosclerosis and OH as a high C-reactive protein levels (CRP).

Atherosclerosis is currently regarded as a low-grade chronic inflammation and maintained by the chronic activation of autoimmune reactions against self-proteins modified by oxidative stress that sustains endothelial dysfunction and plaque development (Profumo et al., 2012). In these terms, a potential OH association with inflammatory cytokines could be expected.

Thyroid hormones influence specific immune responsiveness as well as several aspects of innate and adaptive immunity. However, the relationship between thyroid hormones and immune cells is complex and needs to be clarified with additional investigations. T3 and T4 are able to modulate immune responses through both genomic and nongenomic mechanisms and at physiological concentrations (De Vito et al., 2011).

The acute phase response to inflammation is characterized by the combination of hepatocyte-derived plasma proteins induced by the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) as well as those induced by interleukin-6 (IL-6) (Salvi et al., 2000). A key regulator of the inflammatory response is IL-6, which stimulates the synthesis of acute phase proteins including CRP and fibrinogen.

Moreover, IL-1 has been identified as a chemical mediator released from monocytes/macrophages and exhibits important biologic activity in inflammatory and immunologic responses (Hamaguchi et al., 1991). TNF-α is another important cytokine mediating the induction of adhesion molecules and other cytokines (Salvi et al., 2000) and modulating of the immunologic reactions produced by interferon-γ (IFN-γ) of HLA class II molecules in human thyroid follicular cells (Miyakoshi et al., 1992). Despite the relevance of these molecules in metabolic dysfunctions associated to atherogenesis studies of alternating cytokine levels and OH are still incipient.

Another emerging inflammatory biomarker is the cell-free DNA (cf-DNA) that has been associated with outcome in several conditions as cancer (Schwarzenbach et al., 2011), stroke (Boyko et al., 2011), coronary heart disease (Liu et al., 2015) and reports concerning the outcomes after cardiac arrest that found association of circulating DNA quantities at admission with mortality (Gornik et al., 2014). The possible sources of cell-free DNA in plasma are passive release through cell death (necrosis or apoptosis) and active release by cell secretion (González-Masiá et al., 2013).

Despite the potential influence of OH on blood inflammatory biomarkers there are few studies investigating this potential association. Therefore, we performed here a case-control study that evaluated the association between inflammatory biomarkers and OH. Lipid, prothrombotic and other biochemical markers related with body functions were also evaluated in the sample studied here.

Methods

Study design

A case-control study was performed with eighty subjects enrolled prospectively from clinical laboratory LABIMED, located in Santa Maria-RS, Brazil. Subjects were divided into two groups as follows: control group, 40 healthy subjects (male = 18; female = 22) and newly diagnosed OH group, 40 patients (male = 19; female = 21) without previous pharmacological treatment. OH was defined as TSH higher than 10 mIU/L and low T3 and free thyroxine (fT4) levels (Nanda et al., 2008).

At enrollment, all the participants were tested for serum TSH. When serum TSH levels were higher than 10 mIU/L, we tested fT4, T3, anti-thyroperoxidase antibodies (Anti-TPO Abs) and anti-thyroglobulin antibodies (Anti-TgAbs) levels to evaluate the presence of overt hypothyroidism and if your etiology was autoimmune.

Subjects with previous diseases and dysfunctions that could influence the results were excluded. The exclusion criteria were as follows: subjects taking lipid-lowering drugs, antioxidant vitamin supplements, acetylsalicylic acid, antihistamines, antihypertensive, and exposure to high iodine condition, smokers, alcoholics, pregnant women, women on hormone replacement therapy, diabetics and subjects with acute, chronic or malignant diseases. The protocol was approved by the Human Ethics Committee of the Federal University of Santa Maria (number 2010-87). All subjects gave written informed consent to participate in the study.

Biochemical determinations

Blood samples were collected after 12 h overnight fasting by venous puncture into blue, gray and red top Vacutainers® (BD Diagnostics, Plymouth, UK) tubes. The samples were centrifuged for 15 min at 2500 × *g*, and aliquots of serum were kept at −20 °C for maximum of 4 weeks. Serum TSH, T3, fT4, Anti-TPOAbs and Anti-TgAbs concentrations were measured by chemiluminescent immunometric assay on IMMULITE 2000® (Siemens Healthcare Diagnostics, Los Angeles, USA). Detection limits for TSH was 0.004–14.000 mIU/L, fT4 was 3.9–77.2 pmol/L and T3 was 0.29 nmol/L.

The biochemical markers were spectrophotometry determined using Hitachi U-2800A® equipment (Hitachi High-Technologies Corporation, Japan). High-density lipoprotein cholesterol was measured in the supernatant plasma after the precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride as previously described (Bachorik and Albers, 1986). LDL-C was estimated with the Friedewald equation (Friedewald et al., 1972).

The cytokines IL-1, IL-6, TNF-α and IFN-γ were analyzed using ELISA capture, according to the manufacturer's instructions (Biomyx Technology, San Diego, CA). The D-dimer levels were measured by immunoturbidimetric method on Cobas INTEGRA 400® (Roche Diagnostics, Basel, Switzerland). Fibrinogen levels were measured using coagulation analyzer Sysmex® CA-1500 (Siemens Healthcare Diagnostics, Los Angeles, USA).

The cf-DNA was quantified using PicoGreen fluorescent assay done according to protocol supplied by the manufacturer (Quant-iT™ Picogreen® dsDNA kit, Invitrogen, USA). Picogreen dye was diluted 1:200 with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and incubated with plasma DNA in the dark at room temperature for 5 min. To minimize photo bleaching effects, time for fluorescence measurement was kept constant for all samples. PicoGreen with DNA was recorded at 528 nm using an excitation wavelength of 485 nm. All the fluorescence measurements were recorded on a SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA.

Statistical analysis

Data are presented as mean and standard-deviation (SD). We used Student t test or nonparametric Mann-Whitney *U*-test at the significance level $2\alpha = 0.05$. Multivariate analysis using logistic regression (*Backward wald*) were performed to evaluate if sex, age and body mass index (BMI), variables could to present some influence in the significant results obtained from univariate analysis. To test the potential influence of hormonal status, lipid profile and prothrombotic variables on inflammatory cytokine and cf-DNA levels an additional Pearson correlation test was performed considering just OH subjects. Data were statistically analyzed using SPSS software (version 19.0).

Results

Baseline characteristics of the study (Table 1) showed that, as expected the OH group presented higher TSH and lower T3 and fT4 levels as well as higher levels of autoimmune thyroid markers (anti-TPO and anti-TgAbs). The OH subjects also presented higher levels of total cholesterol (TC), LDL-C and triglycerides, fibrinogen and D-dimer and lower levels of HDL-C than control subjects despite the age and BMI to be similar between groups.

Hereafter proinflammatory cytokines as well as cf-DNA levels were compared between groups. As can see in Table 2, the inflammatory cytokines levels were significantly higher in OH when compared to control subjects. The cf-DNA levels were also higher in OH patients than healthy control subjects. The association between cytokines and cf-DNA with OH was independent of sex, age and BMI variables.

Table 1 – Clinical and laboratory data of study participants.

	Control	OH patients
Age (years)	51.3 ± 10.4	47.6 ± 8.5
BMI (kg/m^2)	24.1 ± 3.5	26.7 ± 3.76
TSH (mIU/L)	1.61 ± 1.07	12.26 ± 2.79*
T3 (nmol/L)	1.64 ± 0.49	0.52 ± 0.13*
fT4 (pmol/L)	85.26 ± 28.38	19.8 ± 0.83*
Anti-TPOAbs (IU/mL)	7.81 ± 6.02	375.3 ± 83.19*
Anti-TgAbs (IU/mL)	7.93 ± 7.60	72.74 ± 14.65*
Total cholesterol (mg/dL)	159. 2 ± 26.74	272.5 ±36.92*
HDL cholesterol (mg/dL)	65.51 ± 14.35	47.5 ±10.13*
LDL cholesterol (mg/dL)	76.71 ± 24.71	195.9 ± 36.81*
Triglycerides (mg/dL)	103.9 ± 28.40	145.5 ± 49.69*
Fibrinogen (mg/dL)	197.9 ± 44.16	388.3 ± 28.9*
D-dimer (ng/mL)	76.9 ± 34.7	169.1 ± 22.24*

Data are expressed as mean ± SD. BMI, body mass index; TSH, thyroid-stimulating hormone; T3, triiodothyronine; fT4: free thyroxine; Anti-TPO Abs, anti-thyroperoxidase antibodies; Anti-Tg Abs, anti-thyroglobulin antibodies.
* Statistically significant.

Table 2 – Inflammatory cytokines and cf-DNA levels in controls and OH patients.

	Control	OH patients
IL-1 (pg/mL)	30.44 ± 6.36	65.33 ± 5.16*
IL-6 (pg/mL)	51.44 ± 5.00	91.08 ± 5.94*
TNF-α (pg/mL)	87.29 ± 5.98	138.9 ± 8.73*
IFN-γ (μg/mL)	120.5 ± 7.71	183.1 ± 9.62*
cf-DNA (ng/mL)	27.25 ± 7.55	37.76 ± 13.80*

IL-1, interleukin-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; cf-DNA, cell-free DNA.
* Statistically significant.

Potential correlation between inflammatory biomarkers and lipids in OH patients was also evaluated (Table 3). In general hormonal status was not associated with cytokine and cf-DNA levels in OH subjects. On the other hand, lipid profile of OH subjects presented important correlations with inflammatory biomarkers studied here. Positive association was observed between total cholesterol and IL-1, IL-6 and IFN-γ cytokines. LDL-C also presented a positive correlation with IL-6 and IFN-γ and triglycerides with IL-1 and TNF-α. Negative correlation between HDL-C and TNF-α cytokine was also observed. Prothrombotic variables did not present correlation

Table 3 – Correlations between total inflammatory cytokines and lipid profile variables in OH patients.

Variables	IL-1 (pg/mL)		IL-6 (pg/mL)		TNF-α (pg/mL)		IFN-γ (μg/mL)	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Total cholesterol (mg/dL)	0.33	0.036	0.46	0.003	–	–	0.47	0.002
HDL cholesterol (mg/dL)	–	–	–	–	−0.45	0.003	–	–
LDL cholesterol (mg/dL)	–	–	0.41	0.009	–	–	0.44	0.005
Triglycerides (mg/dL)	0.35	0.027	–	–	0.40	0.010	–	–

IL-1, interleukin-1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.
Showed only data with correlations statistically significant.

with inflammatory cytokines. cf-DNA levels were not correlated with any variables investigated here.

Considering that lipid profile are highly associated with atherosclerosis and subsequent cardiovascular risk, an additional analysis was performed when the OH patients were categorized in respect of lipid profile cut-off points: total cholesterol (>240 mg/dL), LDL-C (>130 mg/dL), triglycerides (>150 mg/dL) and HDL-C (<50 mg/dL) and the cytokines levels that presented significant correlation were compared. As can see in Fig. 1, the OH with cholesterol >240 mg/dL presented significant higher IL-6 and IFN-γ levels than subjects with lower cholesterol levels. The OH subjects with lower HDL-C levels also presented higher significant TNF-α levels. Again, these associations were independent of sex, age and BMI variables.

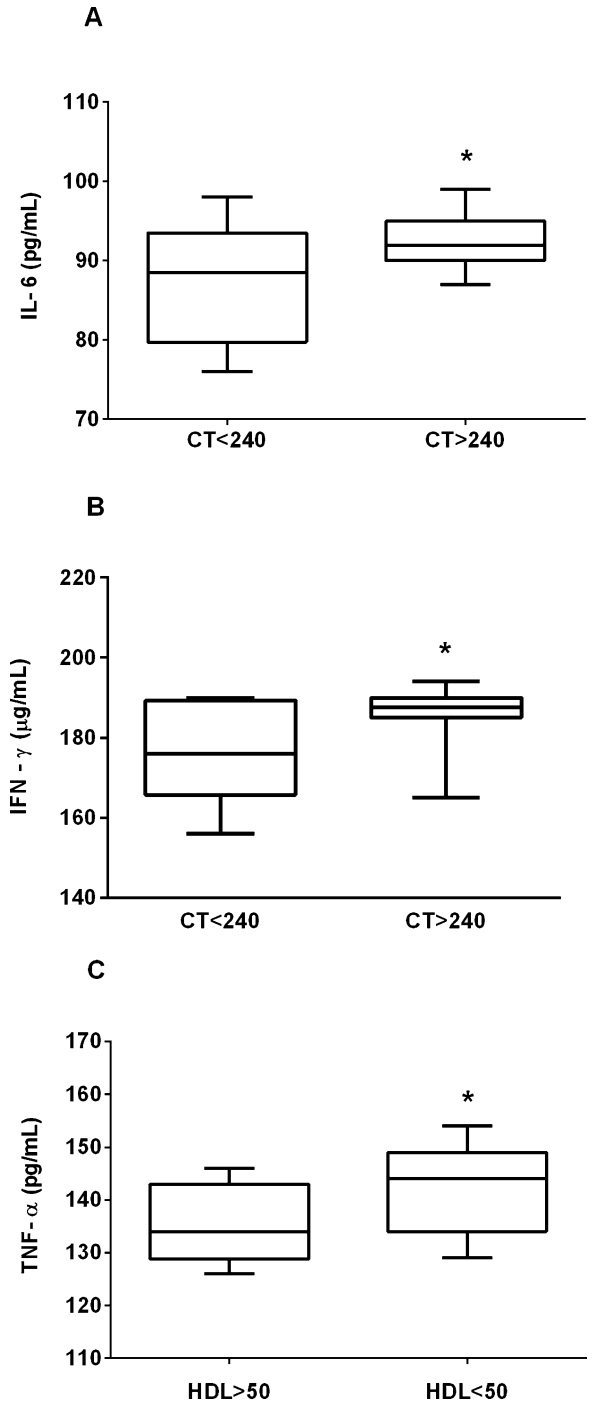

Fig. 1 – Inflammatory cytokines levels according to lipid profile cut-off points in OH patients. (A) Interleukin-6 (IL-6); (B) interferon-γ (IFN-γ) and (C) tumor necrosis factor-α (TNF-α). Cut-off values for elevated total cholesterol (TC) were >240 mg/dL and for decreased HDL cholesterol were <50 mg/dL. * Statistically significant.

Discussion

The present study was performed to evaluate the potential association between OH and blood inflammatory biomarkers (cytokines and cf-DNA) as well as the influence of hormonal status and cardiovascular risk factors know to be associated with OH: lipid profile and prothrombotic variables.

We found higher levels of total cholesterol, LDL cholesterol and triglycerides and lower levels of HDL cholesterol in the OH group. These findings are in agreement with results of other recent investigations, which have showed dyslipidemia associated with hypothyroid status (Lee et al., 2004; Santi et al., 2010; Brenta and Fretes, 2014). The association between lipid profile alterations and OH observed in our study is well established in previous studies and explain, in part, the potential association between hypothyroidism and cardiovascular diseases (Papaioannou et al., 2004; Clausen et al., 2009). A causal mechanism to explain the association between lipid profile alteration and OH is related to the fact that thyroid hormones upregulate the LDL-receptor expression. Thus, the low levels of T3 and T4 found in hypothyroidism promote a reduction in catabolism of lipoproteins leading to hypercholesterolemia (Huesca-Gómez et al., 2002; Mayer et al., 2006).

In addition to the lipid profile altered, hypothyroid dysfunction has been associated with weight gain. In contrast, in the present study BMI not showed significant differences among the groups, although hypothyroid subjects tended to increase (not statistically significant) and presented high atherogenic profile. Similar results were found in literature. Recently, in a study by Belen et al. (2015) no significant difference was observed in BMI between the subclinical hypothyroidism and control groups. Nevertheless, epicardial adipose tissue thickness, that reflects visceral adipose tissue, lipid markers and systolic/diastolic blood pressure were increased in hypothyroidism showing a potential cardiovascular risk factor in these patients. In another study, with hypothyroid women the authors concluded that oxidative stress *per se* had influenced on coronary risk factors independently of BMI (Nanda et al., 2007).

Hypothyroidism has also been associated with alterations in prothrombotic variables. Data reported in the literature have shown higher levels of D-dimer (Chadarevian et al., 2001) and fibrinogen (Gursoy et al., 2006) in patients with overt hypothyroidism. Our results corroborate this association since OH subjects presented higher prothrombotic variables, suggesting a potential hypercoagulable state, which may augment the already existing risk for atherosclerotic complications.

From the confirmation of the association between OH and lipid and prothrombotic alterations, the levels of five

inflammatory biomarkers, four cytokines (IL-1, IL-6, TNF-α and IFN-γ), and cf-DNA levels were performed between case-control groups. The results showed higher levels of these biomarkers in OH subjects indicating the occurrence of a low-grade inflammatory state that is in consonance with atherosclerosis development.

It is widely recognized that OH is associated with increased risk of atherosclerosis. Hyperlipidemia is one of the major risk factors leading to early atherosclerotic vascular diseases. Atherosclerosis has been considered a chronic inflammatory disease, involving both the innate and adaptive immune systems, which modulate the initiation and progression of the lesions, and potentially devastate thrombotic complications (Ross, 1999; Hansson and Libby, 2006). TNF-α along with IFN-γ and IL-1 stimulate IL-6 production by smooth muscle cells. IL-6 gene transcripts are expressed in human atheromatous lesions, and IL-6 is the main hepatic stimulus for CRP production (Blake and Ridker, 2001).

To evaluate if the association between inflammatory biomarkers and OH is a primary event triggered by hormonal status or is a secondary event triggered by lipid and prothrombotic status, a correlation analysis between these variables was carried out considering just OH patients. Results showed clearly that higher cytokine levels present direct correlation with dyslipidemia suggesting that the inflammatory status of OH patients is modulated by lipid profile.

Inflammation is associated with alterations in lipid metabolism that may be mediated by cytokines. The inflammatory profile in subjects with OH is higher in those with TC levels over 240 mg/dL than in those with lower TC levels, as evidenced by the higher IL-6 and IFN-γ levels between the former group of patients. In addition, TNF-α was higher in the OH subjects with HDL-C lower 50 mg/dL. According to these data, we can confirm an association between the degree of hyperlipidemia and inflammatory status in hypothyroid subjects. In respect to the association between lipid metabolism and inflammatory response several experimental studies have demonstrated that the cytokines can affect hepatic enzymes responsible for the synthesis and catabolism of lipids. The administration of TNF-α and IL-1 to rats results in an acute stimulation of hepatic fatty acid synthesis (Feingold et al., 1989). TNF or IL-1 administration to Syrian hamsters increased serum TC levels, and decreased HDL-C levels because cytokines increased hepatic HMG CoA reductase mRNA levels (Hardardóttir et al., 1994). According to these data, we can confirm an association between the degree of hyperlipidemia and inflammatory status in hypothyroid subjects, suggesting the role of inflammatory cytokines in lipid homeostasis.

In this study, the concentrations of cf-DNA were significantly elevated in the OH subjects compared with the healthy controls, suggesting chronic low-grade proinflammatory state and increased death cellular rate in hypothyroidism. Cui et al. (2013) demonstrated that cf-DNA levels are higher in patients with acute coronary syndrome than in patients with stable angina and healthy control, indicating that cf-DNA may be a valuable marker for diagnosing and predicting the severity of coronary artery lesions. Another study suggests that the measurement of cf-DNA may complement troponin and CK-MB in the diagnosis of myocardial infarction (Chang et al., 2003). In very old individuals, plasma cf-DNA level was positively associated with the plasma levels of CRP and IL-1 and inversely associated with the HDL cholesterol level presenting a novel biomarker candidate for systematic inflammation related to aging (Jylhävä et al., 2012). However, we did not observe correlations between the cf-DNA levels and the hormonal, lipid and prothrombotic status. These results indicate that the release of DNA from cells in hypothyroidism is likely to be a result of primary inflammation associated with high lipid levels.

In conclusion, our data suggest that higher lipid concentrations are associated inflammatory status observed in hypothyroidism. Moreover, higher cf-DNA levels and prothrombotic markers could be additional risk factors for atherosclerotic cardiovascular disease in such subjects. However, further studies in a larger sample are required to elucidate the mechanisms of atherosclerosis development in hypothyroidism and provide new strategies for lipid and inflammation management in this disease.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank the LABIMED, BERGMANN, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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