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ORIGINAL ARTICLE

Protective Role of Bilirubin Against Increase in hsCRP in Different Stages of Hypothyroidism

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Abstract In conjunction with thyroxine, bilirubin may play an important role for regulation of hsCRP level and a consequent pro-inflammatory condition in hypothyroidism. In present study we evaluated the dependence of hsCRP changes on total bilirubin (BT) and fT4 level in thirty overt (OH) and thirty subclinical hypothyroidism (SH). Serum BT, hsCRP, thyroxine and TSH were measured in both groups and compared with forty control subjects. Serum values of TSH, hsCRP showed raised ($P < 0.001$ for both) values with lower levels for fT4 and BT ($P < 0.001$ and 0.03 respectively) in hypothyroid patients compared to the controls. ANOVA showed significant increments in TSH and hsCRP values with decreases in fT4 among the control, SH and OH groups respectively ($P < 0.001$). BT values showed decrease in OH group only in comparison to controls ($P = 0.04$). Regression analysis revealed that hsCRP was negatively dependent on fT4 ($\beta = -0.35$, $P = 0.002$) and serum bilirubin ($\beta = -0.40$ and $P < 0.001$ respectively). Univariate general linear model analysis showed this dependence persisted even when carried out distinctly in SH and OH groups separately ($P < 0.001$). TSH did not show any significant predictive value on the hsCRP level in either of these two tests. From these analyses we suggest that serum hsCRP is closely integrated to a lowered synthesis of bilirubin and fT4 in hypothyroid patients. Furthermore, this causal relationship is not only limited to overt but also extends to the SH.

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Introduction

C reactive protein is an acute phase reactant having 206 amino acid residues distributed over five identical subunits [1]. It increases in response to several mediators of inflammation like IL-6, IL-1β released from macrophages and adipocytes [2]. CRP is a pattern recognising protein that identifies phosphocholine on microbes and plays an important role in innate immunity by promoting the opsonin mediated phagocytosis by macrophages. It is an early indicator of immune reaction that starts rising within 6 h of the inflammatory process. As it has a constant half line in plasma, its level in circulation depends on the rate of synthesis, intracellular uptake, and clearance from the body [3]. The role of CRP as a risk factor has been well observed in several disorders like cardiovascular disorders, metabolic syndrome along with type 2 diabetes mellitus, ischemic necrosis and some types of cancers since a substantial time [4–10]. High sensitive CRP (hsCRP) is that low level of CRP that is measured at a level lower than 3 mg/l with more sensitive methods. It is detectable at a much early stage of inflammation and is supposed to be one of the most efficient early indicators of inflammation.

Overt hypothyroidism (OH) has been suggested to alter normal immunological and inflammatory regulatory mechanisms as well. However, significant interplay between the subclinical hypothyroidism (SH), atherosclerosis and the inflammatory markers like TNF-α, NF-kβ and matrix metalloproteinase (MMP) has been reported by Marfella et al. (2012) recently [11]. Role of anti-thyroid globulin and anti-thyroid peroxidase antibodies (TPO-Abs)

are well documented in several studies including some recent ones [12]. CRP has been proposed to indicate the hardness and amelioration of arterial walls thereby precipitating a cardiovascular risk factor in hypothyroidism [13]. Although, some studies reported a significant rise in hsCRP in hypothyroidism, they did not observe a direct relationship between hsCRP with TPO-Abs and autoantibodies against the thyrotropin receptor (TSHR-Abs) [14]. These findings suggested that hsCRP might have an independent role in hypothyroidism other than auto-immune thyroiditis. Studies that reported a persistent increase in the hsCRP level, even after the replacement therapy in SH [15] are in close agreement with this view. However, some studies reported no significant relationship between SH and a raised hsCRP level [16]. Thus, a direct effect of SH on the hsCRP has been still inconspicuous as some studies could not find any significant increase in the hsCRP level in SH compared to control subjects [17].

Bilirubin is supposed to have an antioxidant and anti-inflammatory property within its physiological range that provides it a cardioprotective role [18]. Interestingly, serum bilirubin was found to be a significant negative predictor against development of type 2 diabetes mellitus and cardiovascular disorders among the Korean people [19, 20]. Thyroid hormones have been found to influence both bilirubin synthesis and its transport, and a low thyroxine level is generally associated with a low serum bilirubin [21]. However, the relationship between the changes in the hsCRP and serum bilirubin levels in the spectrum of hypothyroidism encompassing both SH and OH are scarce. Considering all these facts we hypothesized that other than the thyroxine itself, bilirubin might be another major predictive factor for regulation of hsCRP level and a consequent development of the pro-inflammatory conditions in both SH and OH spectrum of hypothyroidism. Accordingly, we designed the present study to evaluate changes of hsCRP and bilirubin level in both OH and SH and to analyze the dependence of hsCRP on major parameters of thyroid function and serum bilirubin level.

Materials and Methods

Study Design

The present study was undertaken as a case control, non-interventional, hospital based study in a tertiary care medical college & hospital of an urban region during the period of December 2013 to June 2014. Both cases and control subjects were selected on the convenience basis from the thyroid clinic of the institution. Both written and informed consents were obtained from the participants as per protocol. The total study protocol followed the guidelines laid in the Helsinki declaration 1975 and modified in 2000 and was conducted after obtaining the permission from the institutional ethical committee.

Selection of Case and Control Groups

Inclusion Criteria

Both case and control subjects were selected from the same geographical area having similar socio-economic background. All patients with raised TSH values were included. Cases with raised TSH but with fT4 levels in the reference range were considered in the SH group whereas, patients with raised TSH and lowered fT4 values were considered as OH. Healthy people accompanying the patients were selected as control subjects after obtaining consents. Both groups were selected within the age group of 20–45 years and were matched for age and sex for each other.

Exclusion Criteria

Persons having any sort of endocrinological disorder other than the thyroid diseases (for the case subjects only), inflammatory disorders, infective disorders or malignant disorders were excluded. Persons with history of smoking, drug intake and alcohol intake were excluded. Subjects with any systemic disorders like hypertension and acute illness were excluded. Any other disorders known to cause rise in hsCRP or an alternation in thyroid status were also excluded from the study. Finally, following the above inclusion and exclusion criteria, thirty (30) cases each in SH and OH groups, and forty (40) subjects in the control group were selected.

Study Parameters

Fasting blood samples were collected from the study subjects in aseptic manner following the appropriate protocol. Serum was separated and was measured immediately for the hsCRP and BT. For measurement of thyroid profile, the serum was stored for a maximum period of 3 days at −20 degree centigrade.

(1) Serum TSH and fT4 were assayed using ELISA kits obtained from Accubind USA. The reference ranges for TSH and fT4 values according to the kit were 0.39–6.16 μIU/ml & 10.32–25.8 pmol/l respectively. The intra- and inter-assay coefficients of variation (CV) for TSH, fT4 were 5 and 6, 3 and 3.7 respectively. The spectrophotometric analysis of thyroid hormones was done on ELISA reader (Tecan, GmbH, Austria, Europe). Based on TSH levels (TSH >6 μIU/ml), these subjects were

classified as hypothyroid (including both the SH and OH), or euthyroid (TSH $\leq$6 μIU/ml). The SH and OH groups were divided depending on the reference range of fT4. Patients having fT4 values within the normal reference range were classified as SH and those having a lower value were considered in the OH groups.

(2) Serum hsCRP was measured by high sensitive immunoturbidimetric method by the immunoturbidity assay kit from ERBA diagnostic, USA. This method was pre-validated with high sensitive nephelometry with a correlation coefficient 'r' value of 0.9952. The inter-run CV as reported by the manufacturer was 3.18 whereas, it was found to be 4.1 in our laboratory set up.

(3) Serum total bilirubin were estimated by the end point Diazo method on fully automated XL 600 Auto Analyzer using standard reagent kits from Erba diagnostics, Manheim, GmbH, Germany.

Statistical Analysis

The data obtained were analysed for significance of difference between the mean values of study parameters between the case and control group by independent t test. The strength of dependence of the hsCRP on study parameters in the case group was assessed by multiple linear regression analysis. The effectiveness of the predictive value for bilirubin level on the hsCRP in SH and OH group separately was assessed by general linear model univariate analysis (ANCOVA). For all statistical analyses the *P* value was considered to be significant at a level of 0.05 or less for 95 % confidence limit. All statistical analyses were performed through the SPSS statistical software version 17.0 for Windows.

Results Analysis

Table 1 shows that TSH and hsCRP values were significantly raised in hypothyroid patients in comparison to the control subjects. fT4 values, as expected, were significantly lower in the hypothyroid group. Although, serum bilirubin value was within the normal range in both groups, it was significantly lower in the hypothyroid patients.

Results of ANOVA were displayed in two tables. In the Table 2 overall intergroup and intra- group differences between the subclinical, clinical and control groups were significant for the TSH, fT4 and hsCRP values, but not for the total bilirubin values. However, the Table 2 could not ascertain the significance of difference between the individual SH, OH and control groups. For this we proceeded to the post hoc ANOVA with Bonferroni correction in the Table 3.

It is evident from the Table 3 that serum bilirubin showed a significant decrease in the OH group only in comparison to the controls ($P = 0.04$). Although, its level in OH group was lower than that found in the SH group, the difference was not significant ($P = 0.532$).

Multiple linear regression analysis was performed to assess the dependence of hsCRP on serum TSH, fT4 and total bilirubin (Table 4). Results revealed that hsCRP was negatively dependent on fT4 and serum bilirubin ($\beta = -0.35$ and -0.40), more significantly on serum bilirubin than the fT4 ($P < 0.001$ and 0.002 respectively). TSH did not show any significant predictive value on the hsCRP level ($P = 0.193$).

To know whether the dependence of hsCRP on serum bilirubin varied between the OH and SH groups, general linear model univariate analysis or analysis of covariance (ANCOVA, Table 5) was performed to assess whether the dependence of hsCRP persisted to be significant on serum bilirubin even when the case population was divided into the separate groups of OH and SH according to their fT4 levels. Results indicated that a significant negative dependence of hsCRP on BT persisted in these two groups even after they were separated into two distinct categories.

Discussion

In the present study, hsCRP level was significantly higher in the hypothyroid patients compared to their levels in normal controls. Moreover, its value in the SH patients, although lower than that found in the OH case group, were significantly higher than that found in the control subjects. Results of the multiple linear regression study also showed

Table 1 Independent t test showing difference in mean values of study parameters between hypothyroid (both SH and OH) and normal subjects

Parameters	Case (n = 60)	Control (n = 40)	t value	*P* value*
TSH in μIU/ml (mean/SD)	20.3/9.2	2.5/1.2	11.0	<0.001
Ft4 (pmol/l)	10.96/3.99	17.93/3.74	−6.4	<0.001
Total serum bilirubin (mg/dl)	0.64/0.3	0.76/0.15	−2.1	0.03
hsCRP (mg/l)	7.4/2.6	3.69/2.3	6.9	<0.001

* *P* value considered to be significant at value of $\leq$0.05 for 95 % confidence interval

that fT4, but not the TSH, was one of the significant predictors of the hsCRP in hypothyroid cases that suggested an inverse dependence of hsCRP level on the cellular function of thyroxine hormones ($\beta = -0.352$, $P = 0.002$, Table 4). Results from ANCOVA (Table 5) revealed that even after separating the hypothyroid patients into two distinct groups of OH and SH based on fT4 level, the dependence of hsCRP on both of these groups existed separately. Keeping the fact in mind that although the fT4 levels were within the reference range for the SH group, it was found in our study that they were significantly lower than that observed in the normal control population (Table 3, post hoc ANOVA). Hence, it can be suggested that a lowered intracellular function of thyroxine was responsible for a rise in hsCRP in these patients. As SH has been found to be associated with the other pro-inflammatory cardiovascular risk factors like dyslipidemia [22] and elevated homocysteine levels [23] in humans and an elevated IL-6, TNF-α, toll like receptor-4 (TLR4), mRNA for TLR4 and NF-kβ in non dyslipidemic rat models [24], it is evident that a lowered intracellular thyroxine function has got a potency to generate low grade systemic inflammation. Our finding that hsCRP is more dependent on the thyroxine level in both OH and SH groups than the TSH level proposes that low grade inflammatory status generated in the hypothyroid patients is so strongly dependent on the thyroxine level that even a lower fT4 level within its reference limit can lead to initiation of a pro-inflammatory condition even at a subclinical stage in the spectrum of

Table 2 One way ANOVA between the study parameters of the subclinical, clinical and control groups

	F	Sig (*P*)*
TSH	73.278	<0.001
FT4	92.815	<0.001
hsCRP	39.134	<0.001
BT	3.196	0.046

* *P* value considered to be significant at value of ≤0.05 for 95 % confidence interval

Table 3 Post hoc ANOVA with Bonferroni correction to show the significance of difference of mean values for individual study parameters among the SH, OH and control groups

Dependent variable	(I) Grouping all	(J) Grouping all	Mean difference (I − J)	Sig (*P*)*	95 % CI	
					Lower bound	Upper bound
TSH	SH	OH	−6.14*	0.004	−10.70	−1.59
		CONT	15.11*	<0.001	10.89	19.32
	OH	SH	6.14*	0.004	1.59	10.70
		CONT	21.26*	<0.001	16.73	25.78
	CONT	SH	−15.11*	<0.001	−19.32	−10.89
		OH	−21.26*	<0.001	−25.78	−16.73
FT4	SH	OH	0.72*	<0.001	0.54	0.90
		CONT	−0.23*	0.003	−0.39	−0.06
	OH	SH	−0.72*	<0.001	−0.90	−0.54
		CONT	−0.95*	<0.001	−1.1	−0.77
	CONT	SH	0.23*	0.003	0.06	0.39
		OH	0.95*	<0.001	0.78	1.12
hsCRP	SH	OH	−2.77*	<0.001	−4.1	−1.2
		CONT	2.60*	<0.001	1.24	3.97
	OH	SH	2.70*	<0.001	1.2	4.1
		CONT	5.31*	<0.001	3.84	6.78
	CONT	SH	−2.60*	<0.001	−3.97	−1.24
		OH	−0.53*	<0.001	−6.7	−3.84
BT	SH	OH	0.10	0.532	−0.08	0.28
		CONT	−0.08	0.65	−0.25	0.08
	OH	SH	−0.10	0.532	−0.28	0.08
		CONT	−0.18*	0.04	−0.36	−0.07
	CONT	SH	0.08	0.649	−0.08	0.25
		OH	0.18*	0.04	0.07	0.37

SH Subclinical hypothyroidism, *OH* Overt hypothyroidism, *CONT* Control

* *P* value considered to be significant at value of ≤0.05 for 95 % confidence interval

Table 4 Multiple linear regression analysis to show the dependence of hsCRP on study parameters in case group (N = 60) when considered together

Model	Unstandardized coefficients		Standardized coefficients	Sig (*P*)*
	B	SD	β	
1				
(Constant)	10.42	1.20		<0.001
TSH	0.042	0.032	0.14	0.193
FT4	−2.12	0.668	−0.35	0.002
TBIL	−3.17	0.81	−0.40	<0.001

* *P* value considered to be significant at value of ≤0.05 for 95 % confidence interval

Table 5 Univariate general linear model analysis (analysis of covariance or ANCOVA) to show the dependence of hsCRP on study parameters (covariates) in subclinical and overt hypothyroid cases classified according to their fT4 values (the fixed variable)

Tests of between-subjects effects					
Dependent variable:hsCRP case					
Source	Type III sum of squares	df	Mean square	F	Sig (*P*)*
Corrected model	204.33[a]	3	68.11	17.20	<0.001
Intercept	359.62	1	359.62	90.84	<0.001
TSH case	10.68	1	10.68	2.70	0.10
BT case	68.28	1	68.28	17.24	<0.001
Grouping fT4	56.45	1	56.45	14.24	<0.001
Error	221.70	56	3.96		
Total	3776.77	60			
Corrected total	426.02	59			

* *P* value considered to be significant at value of ≤0.05 for 95 % confidence interval

[a] R Squared = 0.480 (Adjusted R Squared = 0.452)

hypothyroid disorders. As an explanation of this pro-inflammatory conditions several mechanisms have been suggested like an increased endothelial dysfunction along with a decreased synthesis of endothelium induced nitric oxide synthase (eNOS) due to hyperlipidemia [25, 26], higher levels of IL-6, increased levels of TNF-α etc. [27].

In our present study, serum bilirubin was found to be significantly lowered in our case group in comparison to that in the control population as evident in the independent t test (Table 1). But the results of ANOVA (Tables 2 and 3) revealed that this difference was mainly between the OH group and the normal control group whereas no significant difference was observed between the SH and OH groups. Results of multiple regressions (Table 4) revealed that serum bilirubin emerged as a significant negative predictor of the hsCRP in the hypothyroid group. Moreover, this predictive value persisted when the case population was divided into the SH and OH depending on the fT4 values (Table 5, ANCOVA). Earlier, in cases of subclinical aortic atherosclerosis hsCRP was found to be inversely correlated to the serum bilirubin indicating the protective role of bilirubin against low grade inflammatory process [28, 29]. It was reported that patients with a lower level of serum bilirubin had an eighty percent less chance for developing CVD [30], an increase of 0.1 mg/dl of bilirubin reduced the odds of stroke by nine percent and a ten percent respectively among civilian population and patients with a history of stroke. The anti-inflammatory effects of bilirubin against a rise of hsCRP and CVD have been attributed to its metabolic effects like its antioxidant activity and its inhibitory power to attenuate the oxidation of the low density cholesterol and oxygen radicals [31–33].

Considering the significant dependence of hsCRP on serum bilirubin in both SH and OH patients it can be concluded that an elevation of the pro-inflammatory hsCRP in hypothyroid cases is closely integrated to a lowered synthesis of bilirubin due to a compromised thyroxine activity on the hepatocytes. Furthermore, this causal relationship is not only limited to OH but also extends to the SH spectrum due to a significant dependence of hsCRP on small changes of the thyroxine level. However, limitations of the case control studies must be kept in mind during analysing the final results of the study and we suggest that these results should be validated in future through vertically designed studies involving cohorts of SH and OH groups and observing the effects of thyroxine treatment on the levels of bilirubin and hsCRP in them thereafter.

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Research Involving Human Participants Present study was carried out in strict compliance with the Helsinki Declaration for human studies formulated in 1975 and modified in 2000. The study was started after getting written permission from the Institutional Ethical Committee.

Informed Consent Informed consents were obtained from all individual participants included in the study.

Conflict of Interest The authors Dr. Suparna Roy, Dr. Ushasi Banerjee and Dr. Anindya Dasgupta declare that they have no direct or indirect conflict of interest related to this study. No conflict of interest is related to this study regarding any administrative, financial or authorship matter.

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