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## ASSOCIATION BETWEEN SERUM VALUES OF C-REACTIVE PROTEIN AND CYTOKINE PRODUCTION IN WHOLE BLOOD OF TYPE 2 DIABETIC PATIENTS.

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Running title: C-reactive protein and interleukins in type 2 diabetes

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## ABSTRACT

Diabetes mellitus accelerates the atherosclerotic processes. It is known that inflammation plays a key role in atherosclerosis. The aim of the study was to evaluate in type 2 diabetic patients whether serum levels of C-reactive protein (CRP) is associated to cytokine production in whole blood.

Eighty-nine type 2 diabetic outpatients were enrolled. Blood pressure, body mass index, fasting blood glucose, HbA1c, cholesterol, triglycerides and high sensitivity (hs)-CRP were measured. Interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were measured before and after 24 hours of incubation of whole blood with lipopolysaccharide (LPS) or saline solution.

The basal values of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were low and not significantly related to hs-CRP levels. A univariate analysis showed that the level of IL-1 $\beta$  and IL-6, obtained after 24 hours of incubation of whole blood with LPS, significantly increased with increasing levels of hs-CRP and, after adjusting for potential confounders, IL-1 $\beta$  still remained statistically significant.

In our population of type 2 diabetic patients there is no association between serum hs-CRP levels and the basal levels of IL-6, IL-1 $\beta$  and TNF- $\alpha$ . Conversely, a significant association was observed between serum hs-CRP levels and IL-1 $\beta$  and IL-6 production after 24 hours of incubation of whole blood with LPS. Our data suggest that type 2 diabetic patients with high hs-CRP values might have a major reactivity in response to specific stimuli to produce different interleukins, with possible implications in inflammatory-atherosclerotic processes.

## INTRODUCTION

Epidemiological studies indicate that diabetes mellitus can accelerate atherosclerotic processes and increase the incidence of cardiovascular events and strokes [1]. It has been demonstrated that inflammation plays an important role in atherosclerosis [2] and in the pathogenetic mechanisms of some cardiovascular events [3].

Increasing evidence indicate that the same 'traditional' cardiovascular risk factors, such as hypertension, diabetes, obesity, smoking, and dyslipidemia, might induce a 'low-grade' inflammation state through the stimulation of different cytokines [4-6].

Many 'pro-inflammatory' molecules have been proposed as potential markers for development of cardiovascular events [7]. Among these, the acute phase protein, C-reactive protein (CRP), has been considered as a sensitive marker to predict future cardiovascular events in patients with stable and unstable angina [8,9], and in primary cardiovascular prevention [10], even if recent evidence indicates that CRP does not add substantial predictive value for cardiovascular events beyond established risk factors [11-14]. CRP has been also considered a cardiovascular risk marker in diabetes [15] and an useful marker to predict the risk of developing type 2 diabetes [16,17], indicating that the link between cardiovascular diseases and diabetes might be in part explained by the presence of inflammation.

In the last years it has been shown that in vascular cell cultures CRP stimulates the production of pro-inflammatory molecules, suggesting a direct effect of CRP in atherosclerotic processes. However, its role as a direct etiological agent in atherosclerosis and inflammatory processes remains unclear and under active investigations [18-25].

Recently, it has been demonstrated in healthy subjects that the serum levels of CRP are associated to an increase in proinflammatory molecules and to an increase of IL-1 $\beta$  and TNF- $\alpha$  in LPS-stimulated whole blood [26]. Using an ex-vivo approach, we have investigated whether serum CRP values might influence cytokine production in peripheral blood of type 2 diabetic patients in response to LPS stimulation.

## METHODS

Eighty nine type 2 diabetic outpatients admitted to the Diabetes Unit of Alzano Lombardo, Bergamo, Italy, from January to July 2003 were enrolled. Patients with known malignancy, immunological or inflammatory diseases and recent (< 6 months) myocardial infarction were excluded from the study. All patients gave written informed consent to participate in the study. The study was approved by the local ethic committee.

Patients underwent clinical examination and blood pressure was measured using a mercury sphygmomanometer, after a 5 minute rest. Blood pressure was measured 3 times at 2 minute intervals and expressed as the mean of the 3 readings. Systolic blood pressure was indicated by the first Korotkoff sound and diastolic blood pressure was indicated by the disappearance of Korotkoff sound.

In all patients peripheral whole blood samples were collected from an antecubital vein. Fasting blood glucose, HbA1c, total cholesterol and triglycerides was measured using routinary laboratory tests. CRP was measured using a high sensitivity method (hs-CRP Test, Roche). The lower limit of detection for hs-CRP was 0.03 mg/L. The patients were divided in three groups with respect to the hs-CRP levels, according to the statement of the American Heart Association [7].

Basal values of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  were measured in heparinized whole-blood samples using high sensitivity ELISA (hs-hIL-6, hs-hIL-1 $\beta$ , and hs-hTNF- $\alpha$ ; GE Healthcare-Amersham). The sensitivity of these assays for the three cytokines was 0.1 pg/ml.

In these patients the pro-inflammatory responsiveness was evaluated by measuring the levels of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in whole blood samples after the stimulation of LPS [27,28].

LPS stimulated IL-6, IL-1 $\beta$ , and TNF- $\alpha$  were measured by immunometric assays (Immulite IL-6, IL-1 $\beta$ , TNF- $\alpha$ ), as previously described [29]. The lower limit of detection of these assays were 2 pg/ml for IL-6, 1.5 pg/ml for IL-1 $\beta$ , and 1.7 pg/ml for TNF- $\alpha$ . Aliquots of 3 ml of the same samples

were incubated for 24 hours in sterile conditions at 37°C with saline or LPS (Sigma), at the dose of 100 ng/ml. At the end of this time IL-6, IL-1 $\beta$ , and TNF- $\alpha$  were measured.

### **Statistical Analysis**

Median and interquartile range (1<sup>st</sup> – 3<sup>th</sup> quartile) were used for descriptive purposes due to the skewed nature of the variables considered. A generalized linear model (GLM) was applied to investigate the relationship of LPS-induced IL-1 $\beta$ , IL-6 and TNF- $\alpha$  versus hs-CRP after both log transformation and categorization in the three groups defined above. A multivariate analysis was also performed in order to adjust for the following variables: basal cytokine levels, sex, age at visit, duration of diabetes, BMI, HbA1c, LDL cholesterol, antidiabetic treatment (yes-no), antihypertensive treatment (yes-no), anticholesterol treatment (yes-no) and smoking habits (yes-no). The influence of LPS-induced IL-6, IL-1 $\beta$ , and TNF- $\alpha$  and hs-CRP on the occurrence of cardiovascular events was evaluated by means of a logistic regression model. All tests were done at a significance level of 0.05 and were two-sided.

## RESULTS

Table 1 summarizes the clinical characteristics of the patients. In this sample, in spite of insulin or oral hypoglycemic therapy, blood glucose was not completely normalized. In our population, 43 patients were treated with the association of sulphonylureas and biguanides, 7 with sulphonylureas alone, 7 with biguanides alone, 3 with acarbose, 5 with insulin, and 24 only with non pharmacological treatment. Recommended values of LDL cholesterol for diabetic patients [30] were not obtained. Most of the patients were overweight and 47 (53%) of the patients show LDL cholesterol greater than 100mg/dl.

Twenty patients (23% of the sample) had hs-CRP < 1mg/L (Low hs-CRP group; median 0.6 mg/l, 1<sup>st</sup> – 3<sup>th</sup>quartile: 0.5-0.7 mg/l); 44 patients (49%) had hs-CRP between 1mg/L and 3mg/L (Medium hs-CRP group; median 2.0 mg/l, 1<sup>st</sup> – 3<sup>th</sup>quartile: 1.5-2.3 mg/l), and 25 patients (28%) had hs-CRP > 3mg/L (High hs-CRP group; median 7.7 mg/l, 1<sup>st</sup> – 3<sup>th</sup>quartile: 5.1-11.7 mg/l).

In 66 of the 89 patients (79% of the sample) for whom cytokines have been measured, independently on hs-CRP levels, the basal values of IL-1 $\beta$  were very low, near the sensitivity of the 'high-sensitivity' array (1pg/ml). Basal levels of TNF- $\alpha$  were undetectable, while IL-6 showed a progressive, but not significant, increase according with the increasing levels of hs-CRP (Table 1). After LPS stimulation, we found that the level of IL-1 $\beta$  and IL-6 significantly increased with increasing levels of hs-CRP, while this was not true for the TNF- $\alpha$  production (Table 1). No differences in samples incubated with saline were observed (data not shown). Figure 1 shows the relationship of cytokines with hs-CRP in continuous on the log-scale (A) and the categories of hs-CRP defined above (B). In Fig. 1B we reported the p-values obtained for the comparisons between groups, showing that the increase in IL-1 $\beta$  and IL-6 levels was significant in group 3 with respect to group 1. When a multivariate analysis was performed, adjusting for potential confounders, the

contrast between group 1 and 3 was borderline significant for IL-1 $\beta$  (p=0.048) and became non significant for IL-6 (p=0.117).

During the 3 years follow-up period we have observed the development of 12 cardiovascular events: 5 (33%) in patient with low hs-CRP, 4 (11%) in patient with medium hs-CRP, and 3 (19%) in patient with high hs-CRP. No relation was found between the development of cardiovascular events and the basal hs-CRP levels or LPS-stimulated cytokine levels.

## DISCUSSION

The results of the present study, carried out in type 2 diabetic patients, show an association between the serum levels of hs-CRP and the LPS-stimulated production of IL-1 $\beta$  and IL-6 in whole blood. The basal values of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were low and independent of the hs-CRP measurements. On the contrary, the cytokine production in the whole blood stimulated by LPS tended to be higher for higher values of hs-CRP, with a significant association for the IL-1 $\beta$  synthesis under a multivariate analysis. A weaker association was also shown between LPS-stimulated IL-6 and serum hs-CRP values, while no association was found with TNF- $\alpha$ . These data, obtained by an ex-vivo study in a small population, suggest that type 2 diabetic patients with higher hs-CRP do not show an increase in the basal level of the cytokines considered in this study. These same patients could nonetheless have a predisposition to over-produce the different cytokines in response to a specific stimulus. The evidence that the association between LPS-stimulated IL-6 and serum hs-CRP is lost after multivariate analysis might be explained by a possible interfering action of environmental factors such as lipid composition or glucose control. How CRP and LPS-reactivity may interact remains to be clarified. Infact, it is well known that the production of cytokines induced by LPS depends on the activity of specific Toll-like receptors, such as TLR 4 [31], whereas CRP has to be considered an unspecific marker of inflammation related to IL-6.

In healthy subjects Devaraj et al. demonstrated that basal values of CRP is associated to an increase in some plasma inflammatory mediators and to an increase in LPS-stimulated whole blood TNF- $\alpha$  and IL-1 $\beta$  levels [26]. According to these data, we have found a strong association between serum CRP levels and LPS-stimulated production of IL-1 $\beta$  in whole blood, whereas a weaker association was evident for IL-6. At difference with the data obtained in healthy subjects [26], we could not find any association between serum CRP values and LPS-stimulated production of TNF- $\alpha$  in our patients. We have however to consider that in the study of Devaraj et al. [26] a very stringent exclusion criteria were applied to healthy subjects, including pharmacological treatments.

Conversely, the exclusion criteria in our, most observational study, were limited to the major diseases that may modify the inflammatory parameters. In particular, the 38% of our patients were treated with antihypertensive drugs, mainly angiotensin converting enzyme inhibitors and angiotensin II type 1 receptor antagonists, and the 25% with 3-hydroxy-3-methylglutaryl coenzyme A inhibitors, which are known to interfere with cytokine production [32,33].

*In vitro* studies have suggested that the effects of CRP on inflammatory molecules [19,20] might be dependent on the bacterial contamination or by the presence of sodium azide in the commercial CRP preparation. [21-24]. However, other studies have demonstrated that also sodium azide- and LPS-free CRP promotes endothelial activation and atherothrombosis [25]. In addition, using recombinant human CRP free of sodium azide and bacterial contamination products, it has been demonstrated that CRP, at a concentration known to predict cardiovascular events in stable and unstable angina [8], is also able to increase the expression of the receptor for advanced glycation end products in human endothelial cell cultures, suggesting a link among inflammation, endothelial dysfunction and atherosclerosis in diabetic patients [34].

In rat model of myocardial and cerebral infarction it has been demonstrated that injections of human CRP increases myocardial and cerebral infarct size, through a complement dependent mechanism [35,36]. Recently, the same Authors have demonstrated in rats that the administration of a specific CRP inhibitor, blunted the increase in infarct size produced by human CRP injection, indicating that CRP not only might contribute, through immuno-inflammatory actions, to increase myocardial damage, but also that in this experimental model, the inhibition of CRP might have therapeutical effects [37].

Taken together, these data indicate that the role of CRP as etiological agent in inflammatory processes remains to be proven.

Clinical studies have suggested that the measurements of hs-CRP might be an useful marker for predicting cardiovascular events in different pathological conditions [8-12], and in apparently healthy individuals [38]. Recently, increasing evidence from large epidemiological studies did not

confirm the predictive role of CRP for cardiovascular events, but indicated that CRP measurements add only a little additional predictive value in risk stratification [11-13] .

In our population of type 2 diabetic patients we have observed that serum values of CRP might influence cytokine production in response to LPS, suggesting a predisposition to an over-productions of different cytokines in response to a specific stimulus, with possible implication in inflammatory-atherosclerotic processes.

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## FIGURE LEGENDS

### Figure 1.

A) Relationship of LPS-induced IL-1 $\beta$ , IL-6 and TNF- $\alpha$  versus hs-CRP levels in log-scale. The linear fitted curve is plotted and the p-value for the test on slope is reported.

B) Box and whisker plot of LPS-induced IL-1 $\beta$ , IL-6 and TNF- $\alpha$  by hs-CRP groups. The box includes all the observations between the first and the third quartile, the line in bold and the diamond represents the median and the mean, respectively. Whiskers extend from the edges of the box to 1.5 times the Inter-Quartile Range. P-values for pairwise comparisons are shown inside the figures.

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**TAB. 1. CLINICAL CHARACTERISTICS OF THE TYPE 2 DIABETES PATIENTS BY HS-CRP LEVELS**

	hs-CRP levels							
	Low (n=20)		Medium (n=44)		High (n=25)		Overall (n=89)	
	Median	1 <sup>st</sup> - 3 <sup>th</sup> quartile	Median	1 <sup>st</sup> - 3 <sup>th</sup> quartile	Median	1 <sup>st</sup> - 3 <sup>th</sup> quartile	Median	1 <sup>st</sup> - 3 <sup>th</sup> quartile
<b>Age at visit (yrs)</b>	59.6	54.3-70.0	62.5	57.0-67.5	58.7	50.9-64.0	61.5	54.3-66.6
<b>Duration of Diabetes (yrs)</b>	9.5	3.0-33.0	7.0	2.5-15.0	5.0	1.5-8.0	6.0	2.0-15.0
<b>BMI (Body Mass Index)</b>	25.3	22.1-27.0	26.5	25.2-29.4	28.1	26.7-32.1	26.7	25.2-29.9
<b>Fasting Blood Glucose (mg/dl)</b>	136.5	115.5-167.0	126.0	109.5-153.0	159.0	115.0-213.0	132.0	111.0-177.0
<b>Hb A1C (%)</b>	7.0	6.2-8.0	7.3	6.5-8.5	7.3	6.1-8.7	7.1	6.2-8.5
<b>Triglycerides (mg/dl)</b>	101.0	82.0-134.5	143.5	77.5-177.0	122.0	79.0-163.0	120.0	80.0-167.0
<b>Total Cholesterol (mg/dl)</b>	196.5	172.5-251.0	207.0	175.5-235.0	198.0	180.0-227.0	203.0	175.0-233.0
<b>LDL Cholesterol (mg/dl)</b>	133.5	96.5-161.5	135.0	96.0-147.0	130.0	109.0-147.0	135.0	91.0-151.0
<b>HDL Cholesterol (mg/dl)</b>	50.5	43.0-56.0	49.0	39.0-62.0	44.0	37.0-51.0	47.0	39.8-57.3
<b>Systolic blood pressure (mm Hg)</b>	140.0	130.0-140.0	140.0	130.0-147.5	140.0	130.0-160.0	140.0	130.0-150.0
<b>Diastolic blood pressure (mm Hg)</b>	80.0	80.0-80.0	80.0	80.0-85.0	80.0	80.0-90.0	80.0	80.0-85.0
<b>IL-1<math>\beta</math> (pg/ml)</b>	0.4	0.3-0.9	0.5	0.3-0.8	0.4	0.3-0.6	0.5	0.3-0.8
<b>IL-6 (pg/ml)</b>	5.7	3.0-9.6	6.9	3.5-10.9	10.7	4.3-25.4	7.0	3.5-12.1
<b>TNF-<math>\alpha</math> (pg/ml)</b>	n.d.		n.d.		n.d.		n.d.	
	<b>Yes/No</b>	<b>% (yes)</b>	<b>Yes/No</b>	<b>% (yes)</b>	<b>Yes/No</b>	<b>% (yes)</b>	<b>Yes/No</b>	<b>% (yes)</b>
<b>Antidiabetes Treatment</b>	14/6	70.0	31/13	70.5	20/5	80.0	65/89	73.0
<b>Anticholesterolemic Treatment</b>	5/12	29.4	12/32	27.3	4/20	16.7	21/64	25.0
<b>Antihypertensive Treatment</b>	6/11	35.3	13/31	29.6	13/11	54.2	32/53	38.0
<b>Smoke</b>	4/16	20.0	6/36	14.3	4/21	16.0	14/73	16.0
<b>Sex (F/M)</b>	5/15	25.0	12/32	27.3	8/17	32.0	25/64	28.1

