



08186 Lliçà d'Amunt  
Barcelona  
Spain

Tel.:+ 34 93 860 90 00  
Fax:+ 34 93 860 90 17  
e-mail: biokit@biokit.com  
www.biokit.com

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**SUBJECT** **Publications of studies using  
quantex CRP ultrasensitive**



Quantex CRP ultrasensitive was selected by “Hospital Universitari de Sant Joan de Reus” in Spain in order to perform two extensive clinical and population studies. As a result of these studies, two articles have been published in wellknown scientific journals. We attach the articles herewith:

- 1) Diet and lifestyle are associated with serum C-reactive protein concentrations in a population-based study.  
*J. Lab Clin Med. January 2005*
- 2) Evaluation of a high-sensitivity turbidimetric immunoassay for serum C-reactive protein: application to the study of longitudinal changes throughout normal pregnancy.  
*Clin Chem Lab Med 2005;43(3):308-313*

# Diet and lifestyle are associated with serum C-reactive protein concentrations in a population-based study

NURIA BERTRAN, JORDI CAMPS, JOAN FERNANDEZ-BALLART, VICTORIA ARIJA, NATALIA FERRE, MONICA TOUS, DOLORS SIMO, MICHELLE M. MURPHY, ELISABET VILELLA, and JORGE JOVEN

REUS, CATALONIA, SPAIN

C-reactive protein (CRP) has been proposed as an independent risk factor for cardiovascular disease. In this study we sought to investigate the association between several nutritional and lifestyle factors and serum CRP concentration in a population-based study. We studied 359 individuals (172 women, 187 men; age range 18–75 years) randomly selected from the town hall's registers and assessed their daily dietary intake using a 3-day estimated-food record. The median serum CRP concentration was 1.40 mg/L (range <0.10–47.48 mg/L; geometric mean 1.20 mg/L). We noted significant and independent direct associations between CRP and age, body-mass index, female sex, and serum triglyceride concentration. Bivariate analysis showed a significant inverse association between CRP and many nutrients (e.g., carbohydrates, proteins, lipids, thiamine, pyridoxine, tocopherol, and folate), but multiple-regression analysis indicated that only the effect of dietary folate intake was not dependent on other factors. Differences in folate intake did not produce changes in plasma homocysteine concentration, and we detected no negative correlation between dietary folate intake and log homocysteine ( $r = .02$ ,  $P = .711$ ). Strong positive correlations between the intake of folate and numerous other nutrients were found. This population-based study shows that a higher folate intake, in addition to other known constitutive and lifestyle factors, is significantly associated with a lower serum CRP concentration. (J Lab Clin Med 2005;145:41–6)

**Abbreviations:** ANOVA = analysis of variance; BMI = body-mass index; CRP = C-reactive protein; EDTA = ethylenediaminetetraacetate; HDL = high-density lipoprotein; hs-CRP = C-reactive protein measured with the use of high-sensitivity methods; LDL = low-density lipoprotein; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid

It is generally accepted that vascular inflammation plays a role in the development of atherosclerosis<sup>1,2</sup> and that inflammation seems to be a predictive factor for the further development of acute coronary syndromes such as myocardial infarction and unstable angina.<sup>3</sup> A feature of most forms of inflammation is the

increase in the circulating concentration of several plasma proteins known as acute-phase proteins,<sup>4</sup> among them CRP. Substantial evidence supports a strong relationship between serum concentrations of hs-CRP and cardiovascular diseases and their complications.<sup>5–12</sup> The Physicians Health Study showed that

From the Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan and the Unitat de Medicina Preventiva i Salut Pública, Facultat de Medicina, Universitat Rovira i Virgili, Institut de Recerca en Ciències de la Salut.

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Reprint requests: Jordi Camps, PhD, Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, C/. Sant Joan s/n, 43201-Reus, Catalonia, Spain; e-mail: jcamps@grupsgessa.com.

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participants in the highest quartile of hs-CRP distribution had a 2 times greater risk of stroke, a 3 times greater risk of myocardial infarction or sudden cardiac death, and a 4 times greater higher risk of peripheral-artery disease.<sup>5,6</sup> Many other studies support the hypothesis that baseline CRP concentration is an independent risk factor of future cardiovascular events among apparently healthy individuals.<sup>13</sup> A recent study demonstrated that hs-CRP is a stronger predictor of cardiovascular events than LDL cholesterol concentration, and that adds prognostic information to the data provided by the conventional risk factors.<sup>12</sup> We have studied the effect of several nutritional and lifestyle factors on serum CRP concentration in a population-based study.

## METHODS

**Study population.** A representative sample of 359 subjects from 2 Catalan villages (172 women, 187 men) was randomly selected from the town halls' population registers (mean age 42 years old, range 19–75 years). All the participants were of white-Mediterranean ethnic origin. The sex and age distributions of the subjects selected were in accordance with those of the population registers. We sent 784 subjects, selected randomly, a letter informing them about the study, then contacted them by means of telephone and asked whether wanted to participate. In 15.8% of cases, the letter was sent in errors (eg, subject deceased, wrong address), and 5.1% of addressees were excluded because of dementia, use of folate-antagonist medication, the use of folate or vitamin B<sub>6</sub>/B<sub>12</sub> supplements, or pregnancy. Once these subjects were excluded, 79.1% were eligible for inclusion. Of these, 31.5% refused to participate and 2.6% could not make the appointment. Therefore 65.9% (n = 409) of the subjects deemed eligible participated. All of the selected subjects were invited to attend a clinical examination, to provide a fasting blood sample, and to complete a 3-day dietary record. After the examination and standard clinical laboratory tests, we excluded 50 participants in whom we suspected renal alterations, hepatic damage, or neoplasia. Use of medication was an exclusion criterion in the case interference by vitamins or drugs with homocysteine metabolism (eg, methotrexate, tuberculostatics, theophylline, vitamin B<sub>6</sub> antagonists). The study population did not consume vitamin supplements, and local foods are not fortified with vitamins in Spain. The study was approved by the Ethics Committees of the Hospital Universitari de Sant Joan and the Jordi Gol Foundation. All subjects gave their full informed consent in accordance with the Declaration of Helsinki.

**Methods of dietary assessment.** We assessed dietary intake using a 3-day estimated-food record.<sup>14</sup> Subjects were instructed to record all food and beverage consumption (including snacks) over 2 nonconsecutive weekdays and 1 weekend day. Quantities were expressed in household measures. The food record was checked by the study's dietitian, who verified and classified the quantities and types of food recorded. Portion sizes were confirmed with photographic mod-

els depicting a variety of standard household measures. Each of the records was codified by the dietitian. This was a record of real food-and-beverage intake, not a food-frequency questionnaire, and the subjects registered what they ate rather than estimate their usual eating habits. The French Food Composition Table,<sup>15</sup> complemented by the Spanish Food Composition Table,<sup>16</sup> was used to calculate daily nutrient intake.

**Biochemical measurements.** We obtained blood samples while subjects fasted, collecting specimens into potassium EDTA-containing tubes for homocysteine determinations or into tubes with no anticoagulants added for the other biochemical tests. One week elapsed between recording of food intake and blood sampling. The serum concentration of hs-CRP was measured with the use of a particle-enhanced turbidimetric immunoassay (Quantex hs-CRP kit; Biokit, Barcelona, Spain) with a limit of detection of 0.10 mg/L. Coefficients of variation were less than 2% (intraassay) and less than 4% (interassay). HDL-cholesterol concentration was analyzed by a homogeneous method (ITC Diagnostics, Barcelona, Spain). Serum cholesterol and triglycerides were assessed with the use of standard techniques (ITC Diagnostics). LDL cholesterol was estimated with the use of Friedewald's equation.<sup>17</sup> We measured plasma homocysteine concentration using a fluorescence polarization immunoassay (Abbott Diagnostics, Abbott Park, Ill).

**Statistical analysis.** We used the Kolmogorov-Smirnov and Shapiro-Wilks methods to determine whether the variables were normally distributed. To check differences between CRP quartiles on the variables studied, we used ANOVA or the Kruskal-Wallis method followed by the Mann-Whitney U test, corrected for multiple comparisons when appropriate. To verify the association between CRP and the other variables, we fitted a multiple-linear regression model. This analysis was performed in 2 phases. In the first, we forced the inclusion into the model of factors previously reported to be related to CRP that were also significant in our data (Table I) such as age, smoking, BMI, and triglyceride concentrations. We also included pyridoxine because a significant inverse association has been reported between circulating vitamin B<sub>6</sub> and CRP.<sup>18</sup> Although sex was not significant, it was also included. In the second phase, we determined which nutrients had an independent relationship with CRP, of all the nutrients previously shown to be related (Table I), with the use of stepwise methods of selecting variables. We also forced the introduction of energy intake into the model (a standard procedure to separate the effect of the total amount eaten from the amount of each specific nutrient). CRP concentrations were log-transformed (natural logarithms) before the application of the model. To estimate the degree of change produced in serum CRP concentration by modifications of the independent variable value, we calculated the antilogarithm of the B regression coefficient:  $Average\ percent\ change = (antilog_e B - 1) \times 100$ . For calculation purposes, we assigned CRP concentrations below the assay's limit of detection the value of 0.10 mg/L. Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS) version 11.5 (SPSS Inc., Chicago, Ill).

**Table I.** Nutritional, lifestyle, and biochemical variables classified according to quartiles of serum CRP concentration

Variables	First quartile (<0.1–0.44 mg/L)	Second quartile (0.45–1.38 mg/L)	Third quartile (1.40–3.12 mg/L)	Fourth quartile (3.13–47.48 mg/L)	P
Men (%)	56.2	54.4	51.1	46.7	.595
Age (Y)*	32.8 (12.3)	39.9 (14.6)	44.3 (13.9)	50.9 (14.5)	<.001
Age group (%)					
18–29	46.1	32.2	15.6	10.0	<.001
30–44	32.6	32.2	41.1	23.3	
45–54	15.7	18.9	18.9	30.0	
55–64	3.4	7.8	11.1	11.1	
≥ 65	2.2	8.9	13.3	25.6	
Pack-years (in smokers)*	8.7 (7.6)	14.0 (14.5)	18.3 (17.5)	22.0 (15.5)	.004
Alcohol intake (g/d)*	7.9 (11.8)	9.7 (17.9)	8.3 (14.8)	11.7 (20.9)	.421
Physical exercise in leisure time (% yes)	43.8	54.4	40.0	43.3	.233
BMI (kg/m <sup>2</sup> )*	23.7 (3.3)	25.9 (3.9)	27.8 (4.2)	30.3 (6.1)	<.001
Energy and nutrient intake*					
Energy intake (Kcal/d)	2398 (710)	2347 (735)	2167 (590)	1992 (605)	<.001
Carbohydrates (g/d)	226.3 (75.6)	226.3 (84.4)	204.9 (70.9)	186.8 (69.4)	.001
Proteins (g/d)	99.4 (30.7)	95.7 (27.9)	90.4 (25.5)	83.8 (24.3)	.001
Total lipids (g/d)	114.1 (36.1)	107.1 (36.3)	100.5 (28.0)	90.8 (28.2)	<.001
Saturated fatty acids (g/d)	32.9 (11.9)	30.3 (12.7)	27.9 (9.9)	24.9 (9.8)	<.001
MUFA (g/d)	56.1 (18.4)	53.2 (18.1)	50.4 (14.9)	46.4 (14.7)	<.001
PUFA (g/d)	14.5 (7.9)	13.4 (6.5)	12.5 (5.2)	10.9 (4.1)	.001
Cholesterol (mg/d)	398.7 (170.2)	378.5 (154.1)	371.7 (139.5)	335.9 (127.6)	.041
β-carotene (μg/d)	3864 (3450)	3225 (2711)	2739 (2287)	2897 (2615)	.038
Retinol (μg/d)	372.3 (420.1)	300.2 (376.7)	361.8 (599.9)	312.4 (583.9)	.723
Thiamine (mg/d)	1.54 (0.56)	1.50 (0.52)	1.35 (0.40)	1.20 (0.38)	<.001
Riboflavin (mg/d)	1.87 (0.56)	1.83 (0.58)	4.07 (22.15)	1.58 (0.49)	.399
Pyridoxine (mg/d)	2.05 (0.69)	1.96 (0.65)	1.81 (0.64)	1.71 (0.54)	.002
Cobalamin (μg/d)	5.57 (4.19)	6.41 (6.20)	5.51 (3.72)	5.56 (4.63)	.534
Ascorbate (mg/d)	115.2 (79.1)	97.6 (74.5)	92.8 (64.0)	99.8 (59.8)	.163
Tocopherol (mg/d)	13.9 (7.3)	12.5 (5.9)	12.1 (4.9)	10.6 (3.8)	.002
Folate (μg/d)	323.3 (126.9)	294.0 (107.9)	275.3 (100.3)	270.6 (92.4)	.005
Biochemical measures*					
Cholesterol (mmol/L)	4.86 (0.89)	5.20 (0.96)	5.41 (1.06)	5.65 (0.96)	<.001
Triglycerides (mmol/L)	1.01 (0.53)	1.19 (0.65)	1.49 (0.90)	1.66 (1.48)	<.001
HDL cholesterol (mmol/L)	1.62 (0.41)	1.53 (0.37)	1.50 (0.37)	1.49 (0.38)	.103
LDL cholesterol (mmol/L)	2.79 (0.77)	3.13 (0.86)	3.23 (0.90)	3.40 (0.86)	<.001
Homocysteine (μmol/L)	9.41 (3.71)	9.17 (2.73)	9.26 (3.59)	9.12 (2.32)	.937

\*Quantitative data presented as mean (SD).

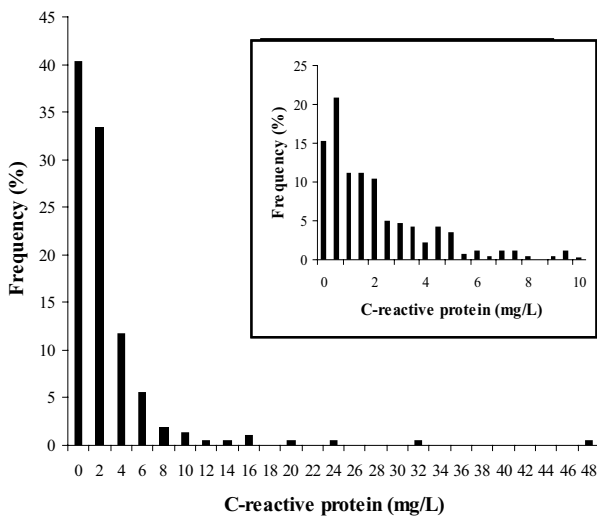
Statistical analysis was conducted with the use of ANOVA or the Mann-Whitney U test corrected for multiple comparisons.

## RESULTS

**Population description.** Of the participants, 140 (39.0%) reported that they were current consumers of alcoholic beverages. Twenty-six other participants (7.2%) reported that they had stopped drinking alcohol between 1 and 38 years ago (4 had not consumed alcohol for 1 year, 12 had not consumed alcohol for 2–10 years, and 10 had abstained for >10 years), and they were therefore considered nondrinkers. Among the previous consumers of alcohol, 4 reported having drunk more than 80 g/day. One-hundred eight participants (30.1%) reported current cigarette smoking. Seventy-eight others (21.7%) reported that they had stopped smoking between 1 and 40 years ago (1 individual had

stopped a year ago, 34 had stopped 2–10 years ago, and 43 had stopped >10 years ago), and were considered as nonsmokers. Among the ex-smokers, 21 reported having smoked more than 20 cigarettes a day. The prevalence of current cigarette smoking was higher among the younger subjects (44% of those aged 18–29 years, 44.8% of those aged 30–44 years, 21.3% of those aged 45–54 years, 16.7% of those aged 55–64 years, and 8.9% of those aged 65–75 years;  $P < .001$ ). Fifty-four women were menopausal, but just 7 took hormone-replacement therapy.

**Serum CRP measurements.** Serum CRP was detectable in 341 of the 359 participants (95.0%). A frequency histogram of CRP concentrations in our popu-



**Fig 1.** Histogram of frequency distributions of serum CRP concentrations in our study population ( $n = 359$ ). The *insert* represents the frequency distributions in the participants with CRP of 10 mg/L or less ( $n = 346$ ).

lation is shown in Fig 1; the distribution was highly skewed. The median CRP was 1.40 mg/L (range <0.10–47.48 mg/L; geometric mean 1.20 mg/L). Thirteen participants (3.6 %) had serum CRP concentrations of 10 mg/L or greater, reflecting an active infectious or inflammatory process.

**Relationship between CRP and nutritional and lifestyle factors.** The results of the nutritional and lifestyle assessment and the biochemical measurements classified according to quartiles of CRP are shown in Table I. Older age, increased BMI, number of pack-years of smoking, and higher serum cholesterol, LDL cholesterol, and serum triglyceride concentration were associated with significantly higher percentages of subjects with CRP concentrations in the highest quartiles. Lower total energy intake was significantly associated with higher CRP values. On bivariate analysis, many nutrients were found to be related to CRP concentration. Lower daily intake of carbohydrates, proteins, and lipids, as well as thiamine, pyridoxine, tocopherol, and folate, were associated with higher serum CRP concentrations.

The combined effects of all the nutritional and biochemical variables on CRP are shown in Table II. When a multiple-regression model was used, only sex, age, BMI, serum triglyceride concentration, and folate intake showed significant and independent associations with CRP. As is shown in Table II, women's serum CRP concentrations were, on average, 29.0 % greater than men's. Being 1 year older was associated, on average, with a 2.2 % higher serum CRP concentration,

**Table II.** Association of nutritional, lifestyle, and biochemical variables with serum CRP concentration

Independent variables	B	Effect (%)*	P
Man vs woman	-.341 (.147)	-29.0	.021
Age (yr)	.021 (.005)	2.2	<.001
Smoking (Y/N)	.018 (.005)	1.8	.895
BMI (kg/m <sup>2</sup> )	.084 (.013)	8.8	<.001
Folate intake (100 $\mu$ g/d)	-.217 (.065)	-19.5	.001
Triglycerides (mmol/L)	.213 (.063)	23.5	.001
Energy intake (kcal/d)	.0001 (.0001)	0.0	.443
Pyridoxine (mg/d)	-.0004 (.147)	-0.01	.998

Goodness of fit:  $F_{(8,350)} = 21.146$ ;  $P < .001$ ;  $R_{\text{corrected}}^2 = .310$ . B-regression coefficient, expressed as mean (SEM).

\*Average percent change produced in serum CRP concentration by the increase in 1 unit of the independent variable value. Multiple-regression analysis introducing variables as described in the Methods section.

and having 1 unit more BMI and serum triglyceride concentration were associated with, on average 8.8 % and 23.5 % higher serum CRP concentrations, respectively. However, a higher dietary folate intake was related to a lower CRP concentration: 100  $\mu$ g/day more was associated with, on average, 19.5% less CRP. We also conducted analyses in which we excluded participants with serum CRP concentrations of 10 mg/L or more and obtained nearly identical results (data not shown).

We detected no negative correlation between dietary folate intake and log homocysteine ( $r = .02$ ,  $P = .711$ ). The relationship between the intake of folate and of other nutrients is shown in Table III. Strong positive correlations between the intake of folate and numerous other nutrients are evident.

## DISCUSSION

This study shows that the frequency distributions, median, and ranges of CRP concentrations in our population from Southern Europe are similar to those previously described for other white populations from Northern Europe and the United States.<sup>4,19</sup> We also confirmed previous findings concerning the association of serum CRP concentration with several variables related to lifestyle.<sup>4,20–23</sup> Although we did not confirm that moderate alcohol consumption reduces the concentration of circulating CRP,<sup>24,25</sup> our results support the findings of a recent study indicating a lack of relationship between alcohol intake and CRP concentration.<sup>21</sup> We believe that the explanation for these discrepancies resides in the higher percentage of participants defined as alcohol consumers in the first study (~90%) compared with the latter study and our own (~40%). Regular physical exercise has been proved to play a

**Table III.** Bivariate relationships between folate intake and other nutrients

Variable	<i>r</i> (n = 359)	<i>P</i>
Energy intake (Kcal/d)	.352	<.001
Proteins (g/d)	.377	<.001
Total lipids (g/d)	.364	<.001
MUFA (g/d)	.429	<.001
β-Carotene (μg/d)	.548	<.001
Ascorbate (mg/d)	.591	<.001
Thiamine (mg/d)	.427	<.001
Pyridoxine (mg/d)	.562	<.001
Cobalamin (μg/d)	.295	<.001

*r* = Pearson's correlation coefficient.

positive effect on endothelial function in patients with coronary-artery disease, but in our study it did not significantly affect CRP concentration.<sup>26</sup>

In this study we extensively investigated the relationship of nutritional factors with serum CRP concentration. To date, little information is available on the subject. Recently Friso et al<sup>18</sup> observed a negative association between plasma pyridoxal phosphate (the active form of pyridoxine) and serum CRP in participants from the Framingham Heart Study. Our results from bivariate analysis show that higher CRP concentrations were associated with lower intake of many nutrients (eg, carbohydrates, proteins, lipids, thiamine, pyridoxine, tocopherol, folate). However, most of these associations were lost in the multivariate analysis, indicating that they were a consequence of other factors, very likely age and sex. We cannot exclude the possibility that socioeconomic status also influences CRP concentration because this parameter is related to nutritional intake.<sup>27</sup> However, a significant correlation of folate intake with CRP persisted after adjustment in a significant and independent inverse relationship.

This positive association of higher dietary folate intake with lower CRP concentration was found in generally healthy and adequately nourished subjects. Investigation of the molecular mechanisms underlying the effects of folate intake on CRP might be an interesting subject for further research. Our findings suggest that these effects are not related to changes in plasma homocysteine concentration. The findings of previous studies have suggested that adequate folate status plays a role in the improvement of endothelial function and the prevention of cardiovascular disease, and they also suggest that some of these effects may be independent of homocysteine concentration.<sup>28,29</sup> Folate appears to improve endothelial function, not only through the activation of the methionine cycle but also through suppression of superoxide production and enhancement of nitric oxide generation.<sup>29</sup> That the lack of correlation

between folate intake and plasma folate and homocysteine concentrations is not surprising in healthy populations must also be taken into account. Intervention trials involving fixed amounts of dietary folate have shown that although the correlation between synthetic folic-acid intake and plasma folate and homocysteine concentrations is high, the correlation between naturally occurring dietary folates and plasma folate concentration is low.<sup>30</sup> Our study was not an intervention study involving fixed amounts of folate from fixed sources, rather, we estimated folate intake from a variety of sources.

We observed that folate intake is highly associated with the intake of several vitamins and other nutrients. Therefore the negative association between folate intake and CRP that we observed in this study may not to reflect a totally independent effect of folate; rather, it may be a result of the combined effect of folates and the other nutrients related to it. Another possibility is that the observed effect is simply the result of the simultaneous intake of other nutrients and that folate intake is a marker of the effect. The folates ingested by this population were mainly derived from 3 major food groups: 30% from vegetables, 17% from cereals, and 16% from fruits. These results are in agreement with those of a recent study showing that frequent fruit and vegetable intake is associated with lower CRP concentrations in the elderly.<sup>31</sup> These foods are also rich sources of other vitamins and minerals whose effects on CRP are not known. Therefore we can interpret folate intake as a marker of a dietary pattern that affects CRP concentration.

In summary, this population-based study shows that higher folate intake, in addition to other known constitutive and lifestyle factors, is significantly associated with lower serum CRP concentration.

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# Evaluation of a high-sensitivity turbidimetric immunoassay for serum C-reactive protein: application to the study of longitudinal changes throughout normal pregnancy

Núria Bertran<sup>1</sup>, Jordi Camps<sup>1,\*</sup>, Joan Fernández-Ballart<sup>2</sup>, Michelle M. Murphy<sup>2</sup>, Victoria Arijá<sup>2</sup>, Natàlia Ferré<sup>1</sup>, Mònica Tous<sup>1</sup> and Jorge Joven<sup>1</sup>

<sup>1</sup> Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan,

<sup>2</sup> Unitat de Medicina Preventiva i Salut Pública, Facultat de Medicina i Ciències de la Salut, Universitat Rovira i Virgili, Institut de Recerca en Ciències de la Salut, Reus, Catalunya, Spain

## Abstract

C-Reactive protein has been associated with several complications of pregnancy. The aims of the present study were: (1) to evaluate a turbidimetric immunoassay for the measurement of C-reactive protein; and (2) to investigate the chronological changes of the levels of this protein from preconception throughout normal pregnancy and its relationship with variables associated with preconception and pregnancy outcome. Inter-assay imprecision was <5% for C-reactive protein >1 mg/L and 18% at a mean value of 0.33 mg/L. The limit of detection was 0.10 mg/L. The method was linear between 0.10 and 30 mg/L. There were no observed interferences from jaundice, hemolysis, lipemia or paraproteinemia at the levels studied. There was good agreement with the nephelometric method. A total of 39 women were studied at preconception, at 8, 20 and 32 weeks of pregnancy, and in labor. Preconception C-reactive protein concentration was  $1.17 \pm 0.18$  mg/L and increased ( $p < 0.001$ ) throughout pregnancy up to  $5.69 \pm 0.82$  mg/L. Body mass index at preconception and weight gain during pregnancy were the main factors associated with this increase in C-reactive protein.

**Keywords:** C-reactive protein; inflammation; method evaluation; pregnancy.

## Introduction

C-Reactive protein (CRP) has been associated with several diseases, involving endothelial dysfunction and systemic inflammation, such as type 2 diabetes mellitus, metabolic syndrome, and cardiovascular dis-

ease (1–10). Endothelial dysfunction and inflammation are involved in the pathogenesis of preeclampsia and other important complications of pregnancy, including gestational diabetes and fetal overgrowth (11–13). Hence, its measurement has recently aroused considerable interest. Several studies have analyzed serum CRP concentrations at different stages of pregnancy in order to investigate its association with various pregnancy complications (12–16). However, appropriate interpretation of these studies should be based on knowledge of the temporal changes in serum CRP concentrations during normal pregnancy, which have not yet been reported.

The development of high-sensitivity CRP assays has played a pivotal role in the exploration of its involvement in endothelial dysfunction and subclinical inflammation (17). These methods allow the reliable measurement of low concentrations of serum CRP and are often easily automated, which permits the investigation of serum CRP in healthy populations.

The aim of this study was: 1) to evaluate a particle-enhanced turbidimetric immunoassay (PETIA) for the measurement of CRP concentrations; and 2) to investigate the chronological changes in the levels of this protein from preconception throughout normal pregnancy, and its relationship with pre-pregnancy and delivery maternal body mass index (BMI), age, parity, weight gain during pregnancy, and fetal and placental weight at birth.

## Materials and methods

### Assay method

The CRP PETIA was performed with an ILab 1800, an automated, random-access, discrete clinical chemistry analyzer (Instrumentation Laboratories, Milan, Italy). This instrument allows spectrophotometric and turbidimetric reactions to be carried out at 37°C, using one or two reagents, in bar-coded primary tubes. We used commercial reagents obtained from Biokit (Quantex hs-CRP kit, Biokit, S.A., Barcelona, Spain). The assay requires two reagents. In the first step, 6 µL of serum was diluted with 130 µL of 0.17 mol/L glycine buffer (pH 9.0) with sodium azide (0.8 g/L) and incubated for 192 s. In the second step, the diluted sample was mixed with a suspension of 130 µL of uniformly sized polystyrene latex particles (approx. 180 nm in diameter) coated with anti-human CRP monoclonal antibody, and incubated for 324 s. The resulting agglutination was read at 570 nm. Both reagents are liquid, require no preparation before the analysis and are stable at 4°C for up to 1 year. The linearity claimed by the manufacturer ranged between 0.25 and 20 mg/L.

\*Corresponding author: Dr. Jordi Camps, Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, C/ Sant Joan s/n, 43201-Reus, Catalunya, Spain  
Phone: +34-977-310300, Fax: +34-977-312569,  
E-mail: jcampes@grupsgassa.com

Values higher than 20 mg/L were automatically diluted one-third and re-run, so linearity could be expanded to 60 mg/L.

### Calibrators and controls

The PETIA assay calibrators were supplied by Biokit (Quantex ultrasensitive CRP standard multipoint) and consisted of five vials of liquid standards from human sources ranging from 0.50 to 20.00 mg/L. These calibrators have been standardized according to Certified Reference Material 470 from the European Community Bureau of Reference (Institute for Reference Materials and Methods, Geel, Belgium). The analyzer automatically performs calculation of results by point-to-point interpolation. The control (CRP range 1.24–1.87 mg/L) was also from Biokit. Four pools of sera designated A–D and with increasing CRP concentrations were used for performance studies. The sera were obtained from routine clinical samples, mixed and then frozen. When necessary, the samples were thawed and mixed gently for 20 min on a rotating mixer.

### Imprecision study

Intra-assay imprecision was determined with 20 replicate analyses of the control and the four pools. To assess inter-assay imprecision, aliquots of these controls stored at  $-20^{\circ}\text{C}$  were analyzed over 20 consecutive days.

### Recovery

We added 20  $\mu\text{L}$  of three different calibrators (20.00, 10.00 and 5.00 mg/L) to 180  $\mu\text{L}$  of a sample containing undetectable levels of CRP.

### Linearity and detection limit

Linearity was assessed by measuring serial dilutions of three high samples (valued at 22.3, 24.8, and 31.5 mg/L) in physiological saline solution, from undiluted up to a dilution of 1/128. The assessment of linearity assumed that the analyzer automatically diluted one-third of the high samples. The detection limit was determined by measuring the absorbance of the reagent blank 20 times. The mean and SD were calculated and the detection limit was defined as the CRP concentration for an absorbance equal to the mean of the reagent blank value  $+2$  SD.

### Interference

Interference from triglycerides, hemoglobin, and bilirubin was assessed as previously described (18). Pools B, C and D were supplemented with chylomicrons, hemoglobin, or bilirubin at various concentrations, and the percentage recovery was then calculated. Experiments were carried out in triplicate.

### Effect of paraproteinemia and rheumatoid factor

The effect that non-specific aggregation and/or cross-reactivity from paraproteinemia or rheumatoid factor had on the CRP determination was assessed as described elsewhere (18). Serial dilutions (300  $\mu\text{L}$ ) in physiological saline of sera from patients with immunoglobulin (Ig)G, IgM, and IgA myeloma or rheumatoid arthritis were added to identical volumes of pool C, gently mixed on a Coulter mixer (Coulter Electronics Ltd, Hialeah, FL, USA) for 20 min and analyzed. The percentage recovery was then calculated. Assessments were carried out in triplicate.

### Method comparison

The PETIA CRP method was compared with an immunonephelometric measurement on a Behring nephelometer II (Dade-Behring, Liederbach, Germany). The latter technique was taken as the reference method. The manufacturer's claimed analytical range for the Behring method was 0.17–210 mg/L. The limit of detection was 0.01 mg/L. Intra- and inter-assay CV were 4.1% and 7.5%, respectively. Method comparison was performed on 100 serum samples (55 men, 45 women, aged from 21 to 65 years) obtained from routine analysis requests, and covering a wide range of CRP concentrations. The use of leftover materials for evaluation of diagnostic methods is in agreement with the European Law for Medical and Diagnostic Products. These samples were analyzed double blind in parallel on both instruments.

### Participants

A total of 39 healthy women aged between 18 and 35 years with the intention of immediately becoming pregnant participated in the study. They were a subgroup of a more extensive longitudinal study on the evolution of women's nutritional status before conception and throughout pregnancy, which was carried out in the Unit of Preventive Medicine and Public Health of our university. The design and background to this study have been explained in detail previously (19). The approval of the Ethics Committee of our hospital was obtained and all participants gave their signed informed consent in accordance with the declaration of Helsinki.

On the week of each blood sample collection, subjects were weighed (kg) using scales with an accuracy of 0.1 kg. At preconception their height (m) was also measured to an accuracy of 0.1 cm. Their BMI at preconception was then calculated as  $\text{weight}/\text{height}^2$ . Skinfold measurements at the triceps, biceps, suprailiac and subscapular regions were performed at preconception and the last pregnancy visit and used to calculate the percentage fat mass as previously described (20). Blood samples were drawn from the antecubital vein at preconception (2–10 weeks before conception), at 8, 20 and 32 weeks of pregnancy, and on admission to hospital with confirmed labor. All blood samples were fasting with the exception of those taken during labor. Serum for CRP measurement was stored at  $-80^{\circ}\text{C}$ .

### Statistical analysis

We used standard methods (Kolmogorov-Smirnov and Shapiro-Wilks) to check whether variables were normally distributed when required for the application of a statistical test. For method evaluation, values of CRP by the two assays were compared using the Wilcoxon rank-sum test. The association between variables was measured using Deming regression analysis (21). The degree of agreement between the turbidimetric and nephelometric methods was estimated using the Bland-Altman graphical procedure (22). A one-way analysis of variance (ANOVA) for repeated measures was used to explore the changes in CRP concentrations over pregnancy time (intra-subject factor). A repeated contrast (comparing the mean at each time point with the mean at the subsequent time point, maintaining the type I error at 5%) was used to test the significance of differences in CRP means, between the different time points of pregnancy, labor and preconception. A multiple linear regression model was fitted to test the effect of maternal age, parity, preconception BMI, gestation length, weight and fat mass gain during pregnancy, newborn sex, and birth and placenta weight on CRP. Diagnosis of residuals was used to test whether the

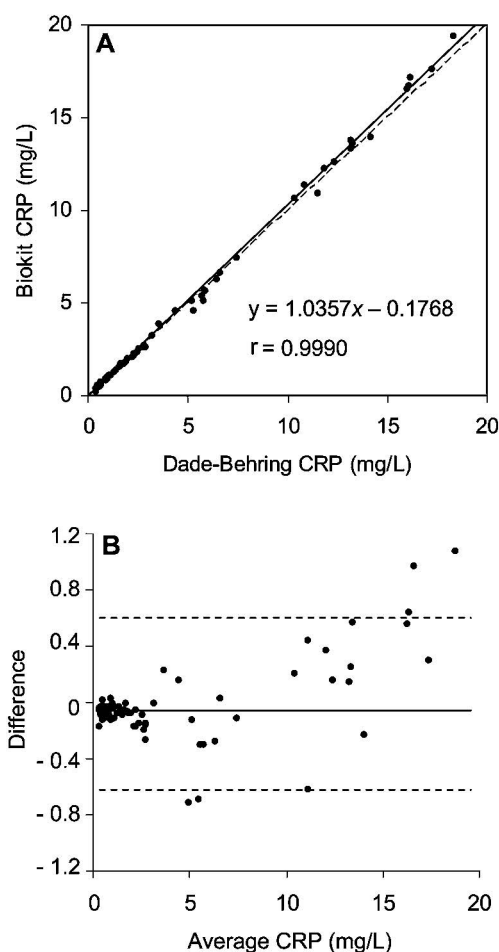
**Table 1** Analytical imprecision of the CRP PETIA.

Control material	Mean CRP, mg/L	Intra-assay		Inter-assay	
		SD, mg/L	CV, %	SD, mg/L	CV, %
Commercial	1.40	0.04	2.86	0.06	4.29
Pool A	0.33	0.05	15.15	0.06	18.18
Pool B	2.18	0.05	2.29	0.10	4.58
Pool C	10.80	0.10	0.92	0.19	1.76
Pool D	17.34	0.13	0.75	0.26	1.50

**Table 2** Effects of paraproteinemia and rheumatoid factor on the PETIA for CRP.

Constituent	Recovery, %			
	1:2 dilution	1:4 dilution	1:8 dilution	1:16 dilution
IgG (54 g/L)	101 (5)	100 (3)	100 (2)	99 (2)
IgA (40 g/L)	103 (3)	100 (2)	101 (6)	104 (2)
IgM (36 g/L)	100 (4)	101 (5)	103 (5)	107 (4)
RF (800 kIU/L)	100 (3)	99 (2)	98 (5)	100 (5)

Mean (SD) percentages of recovery (n=3) after addition of serial dilutions of sera from three patients with myeloma and one patient with rheumatoid arthritis to aliquots of pool C. RF, rheumatoid factor.



**Figure 1** (A) Deming regression analysis and (B) Bland-Altman plots for CRP measurements by the Biokit and Dade-Behring methods. In (A) the dashed line represents the line of identity; in (B), the 95% limit of agreement.

assumptions for the regression model were fulfilled. We refused the null hypothesis when the p-value was equal to or lower than 0.05. Except when the contrary is indicated, results are expressed as means and SD. Version 11.0 of the Statistical Package for Social Sciences (SPSS) for Windows was used for data analysis.

## Results

### Assay performance

Intra- and inter-assay coefficient of variation (CV) values are shown in Table 1. They were lower than 10%, except for pool A (CRP 0.33 mg/L), which had values of 15% and 18%, respectively. The percentage recovery from triplicate measurements was  $99.0 \pm 7.0\%$ . The assay was linear over the range studied. The regression line for observed vs. expected values was  $y = (0.989 \pm 0.043)x - (0.06 \pm 0.23)$  ( $r = 0.999 \pm 0.001$ ). The detection limit was 0.10 mg/L. There was no substantial interference from hemoglobin ( $\leq 5$  g/L), jaundice (bilirubin  $\leq 175$   $\mu\text{mol/L}$ ), or hyperlipidemia (triglycerides  $\leq 5.00$  mmol/L). We did not observe any significant interference from paraprotein IgG up to 27 g/L, IgM up to 18 g/L, IgA up to 20 g/L, or rheumatoid factor up to 400 kIU/L (Table 2).

### Method comparison

For 100 samples, the median value for CRP was 2.02 mg/L (interquartile range 0.85–5.74 mg/L) for the Biokit assay and 2.20 mg/L (interquartile range 0.90–6.05 mg/L) for the Dade-Behring assay. These differences were statistically significant ( $p = 0.012$ ) and indicated a slight underestimation by the Biokit method. Figure 1A shows the results of Deming regression analysis. There was direct significant asso-

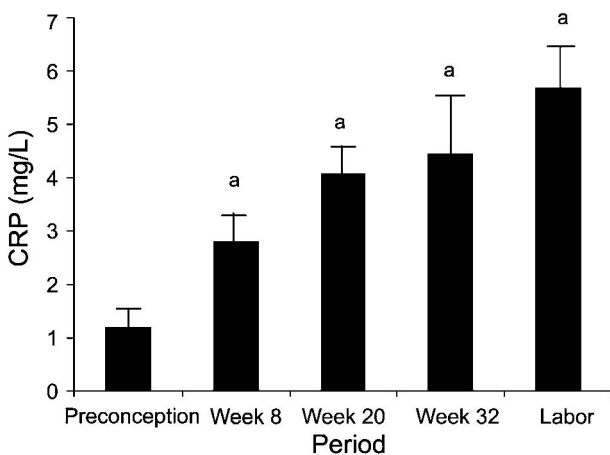
**Table 3** Baseline and delivery characteristics of women (n=39) and their neonates.

	Mean (SD)	Frequency, %
Age, years	29.2 (2.7)	
Parity		
0		66
1		34
BMI (preconception)	23.3 (3.1)	
Gestational age, weeks	40.0 (0.93)	
Weight gain, kg	11.2 (3.1)	
Fat mass variation, kg	0.82 (2.13)	
Child sex		
Male		39
Female		61
Birth weight, g	3387.6 (505.9)	
Placenta weight, g	673.5 (140.9)	

ciation between both measurements ( $r=0.999$ ; slope, 1.03 with SE  $5.71 \times 10^{-3}$ ; y-intercept,  $-0.17$  with SE  $3.89 \times 10^{-2}$ ). Figure 1B is a Bland-Altman plot showing the degree of agreement between both methods. The average difference was  $-0.02$  mg/L (95% confidence interval  $-0.07$  to  $0.04$  mg/L). Maximum absolute difference between methods was 1.2 mg/L at a mean CRP concentration of 18.83 mg/L.

### Study in pregnant women

Baseline and delivery characteristics of all participants are presented in Table 3. Serum CRP concentration before conception was  $1.17 \pm 0.18$  mg/L. This parameter significantly increased ( $p < 0.001$ ) throughout pregnancy up to  $5.69 \pm 0.82$  mg/L at labor (Figure 2). The combined effect of the variables studied on CRP at different periods was analyzed by multiple linear regression analysis and is shown in Table 4. There was a borderline relationship ( $p=0.057$ ) between mean BMI and CRP during the preconception period. The relationship between preconception BMI and CRP during pregnancy was clearly significant ( $p=0.001$ ) at 8 and 20 weeks, but disappeared by week 32 and during labor. Weight gain was significantly associated with serum CRP concentrations at week 8 ( $p=0.037$ )



**Figure 2** Serum CRP concentrations (mean and SD) in women at preconception and throughout pregnancy. <sup>a</sup> $p < 0.001$  with respect to preconception.

and total weight gain during pregnancy with CRP during labor ( $p=0.046$ ). None of the other variables showed any significant relationship with serum CRP concentrations in any of the periods of investigation.

### Discussion

The analytical performance of the PETIA evaluated in the present study shows that this method is comparable to most reported high-sensitivity CRP turbidimetric immunoassays (23–27). Imprecision was  $< 5\%$  for all the control materials studied, except for pool A (CRP 0.33 mg/L), which was 18%. In this respect, our results differ from those previously published for this method (25) and indicate that sensitivity would need to be improved to accurately measure CRP concentrations in the lowest range. The detection limit was 0.1 mg/L. Functional sensitivity [CRP concentration corresponding to a 20% CV; defined in ref. (27)] was 0.30 mg/L. Recovery was close to 100% and linearity was excellent over the entire measurement range. Lipemia, jaundice and hemolysis appeared to have little influence on the assay. We did not observe any interference from rheumatoid factor nor from paraproteinemia, irrespective of whether the paraprotein was IgG, IgA or IgM. For agreement studies, we compared the results obtained with our PETIA with those obtained with a Behring II nephelometer. The latter method has been clinically validated (27). We observed good agreement between both methods, with differences between both methods not higher than 10%.

Using the PETIA we performed a prospective study to investigate the chronological changes in serum CRP concentration before and throughout normal pregnancy. The study of inflammatory markers in pregnancy has received considerable attention from researchers recently. Tjoa et al. (15) reported an association between high CRP concentrations during the first trimester and the subsequent development of preeclampsia or the delivery of a growth-restricted baby. Keski-Nisula et al. (28) reported that CRP concentrations were higher in parturient mothers who presented with cesarean section with membrane rupture than in those with intact membranes. Hvilsom et al. (29) observed that high CRP concentrations at the beginning of pregnancy were associated with a two-fold increased risk of preterm delivery. These results highlight the importance of appropriate understanding of the changes in serum CRP concentrations in normal pregnancy. Several investigations concluded that BMI is the main determinant of CRP in both lean and obese pregnant women (13, 14, 30). However, all of these were cross-sectional studies, which limits the applicability of their findings to different stages of pregnancy. Ours, on the other hand, had a prospective, longitudinal design including the preconception period, which allowed measurement of the increase in serum CRP throughout pregnancy and the investigation of factors that could influence it. We observed that maternal weight was the main deter-

**Table 4** Association of the variables studied with serum CRP concentration (raw data) at preconception and throughout pregnancy.

Independent variable	Pre-conception		Weeks of pregnancy						In labor	
			8		20		32			
	B	p	B	p	B	p	B	p	B	p
Age, year	0.004 (0.052)	0.947	0.035 (0.159)	0.828	0.221 (0.164)	0.189	0.287 (0.455)	0.533	0.340 (0.357)	0.349
BMI (preconception, kg/m <sup>2</sup> )	0.091 (0.046)	0.057	0.504 (0.141)	0.001	0.498 (0.136)	0.001	0.189 (0.347)	0.591	0.194 (0.288)	0.506
Weight gain from previous visit, kg			0.794 (0.365)	0.037	0.219 (0.523)	0.679	0.164 (0.610)	0.790		
Parity (one previous child)					0.456 (0.886)	0.611	-1.184 (2.844)	0.680	-1.136 (2.437)	0.645
Gestational age, week									-0.275 (1.147)	0.812
Total weight gain, kg									0.671 (0.319)	0.046
Fat mass variation, kg									0.165 (0.468)	0.727
Child sex (female vs. male)									-0.800 (1.983)	0.690
Birth weight, g									0.001 (0.003)	0.772
Placenta weight, g									0.003 (0.008)	0.695
Goodness of fit, F	1.937	0.159	5.702	0.003	6.123	0.001	0.189	0.942	1.183	0.349
R <sup>2</sup> <sub>corrected</sub> × 100	5.0		28.7		37.6		2.5		4.8	

B, regression coefficient as mean and standard error (SE).

inant of serum CRP concentration in normal pregnant women, with preconception BMI playing a predominant role during the first half of pregnancy, and the total weight gain being more important in labor.

The relationships between CRP and measurement of body weight and obesity are consistent with that of the in vivo release of IL-6 from adipose tissue (4). It is known that nearly one-quarter of IL-6 produced in vivo originates from adipose tissue and is thought to modify adipocyte glucose levels, lipid metabolism, and body weight (27). In conclusion, these data support the hypothesis of pregnancy as a proinflammatory condition related to weight gain.

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