Chapter 2

High-sensitivity C-reactive protein methods examined

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Abstract

Background

High sensitivity CRP (hs-CRP) is considered as an important risk factor for coronary heart disease. Until now hs-CRP has primarily been used for research purposes, but implementation, as a routine laboratory parameter is obvious. In daily practice one CRP method giving reliable results both in the low and high concentration range is to be preferred.

Methods

A new hs-CRP method developed for the Beckman Coulter IMMAGE®, called CRPH, and measured by near infrared particle immunoassay (NIPIA) was evaluated and compared to the IMMULITE hs-CRP method from Diagnostic Products Corporation and to the BNA hs-CRP method from Dade Behring. In the higher range the hs-CRP methods were also compared to our routinely used CRP method performed by immunoturbidimetry on the Synchron LX®20. Besides, hs-CRP IMMAGE method was also compared to the current IMMAGE® CRP method (all three from Beckman Coulter).

Results

In the lower range, 0.2 – 10 mg/L, the CRPH IMMAGE® method was linear down to 0.2 mg/L and not influenced by turbidity. Total imprecision (CV) was less than 10% in the range from 0.2 – 40 mg/L. The CRPH IMMAGE® method showed good agreement with BNA and IMMULITE hs-CRP methods.
Above 100 mg/L Bland Altman plots showed highly scattered data for all CRP comparisons.

Conclusion

The new IMMAGE® hs-CRP is a good method for clinical risk stratification. However, for all CRP and hs-CRP methods poor correlations are seen in the upper part of the manufacturers claimed reference ranges. Therefore, at present the use of two different methods, a hs-CRP for risk stratification and a normal routine CRP method, is required.

Introduction

C-reactive protein (CRP) is a valuable laboratory parameter in the clinical evaluation of infection, inflammation or trauma. Several clinical studies have demonstrated that this acute phase protein also has prognostic value in patients with acute coronary syndromes and is moreover a strong independent predictor of future coronary events in apparently healthy subjects. Rifai and Ridker recently reviewed clinical studies on CRP as a prognostic parameter in coronary heart disease[1].

In most laboratories CRP measurements are performed by immunoturbidimetry on routine clinical chemistry analyzers, having detection limits for CRP of 2 - 5 mg/L. For studies on CRP as a risk factor for coronary artery disease, a high-sensitivity CRP (hs-CRP) method is required. A variety of methods have now been introduced for measuring hs-CRP and Roberts *et al.* recently compared the different methods[2,3]. In their first study, comparing four different methods, both normal and increased values in the range of 0 – 250 mg/L, were compared[2]. In their second study, nine hs-CRP methods were compared in 388 apparently healthy individuals and comparisons were focused on values in the range of 0 – 10 mg/L[3]. Hs-CRP assays should indeed distinguish the minor increases from the upper limit of normal. However, until now hs-CRP has primarily been used for research purposes and introduction, as a routine laboratory parameter, is obvious. The laboratory has to be sure to report reliable CRP results regardless of the clinical context; therefore also for the upper part of the manufacturers claimed measuring ranges (varying from 0.2 to 500 or 1000 mg/L), agreement between hs-CRP and CRP methods is required.

We report here the evaluation of a new hs-CRP method for the Beckman Coulter IMMAGE. This new method is compared to two hs-CRP methods (IMMULITE and BNA) and in the higher range also to our routine CRP method, on the routine clinical chemistry analyzer LX20 from Beckman Coulter as well as the current IMMAGE CRP method.

Materials and methods

Samples

Venous blood samples were collected from 291 ostensibly healthy blood donors, 177 male and 114 female, presenting at the Sanguin Blood Bank in Maastricht. Samples for method comparison were collected from 521 patients in whom a CRP was requested for routine analysis. The Medical Ethical Committee of the Hospital approved the procedure followed.

Serum was separated from the red cells by centrifugation and stored at -70^{o}C until analysis[4]. There was no freeze-thawing effect as reported previously[5].

Methods

Hs-CRP on the IMMULITE Automated Analyzer from Diagnostic Product Corporation is a two-site chemiluminescent enzyme immunometric assay, with one ligand labeled anti-CRP monoclonal antibody and an alkaline phosphatase-labeled anti-CRP polyclonal antibody (Diagnostic Product Corporation, Los Angeles, CA, cat no LKCR1).
Hs-CRP on the BNA nephelometer from Dade Behring is performed by particle enhanced immunonephelometry, using a monoclonal antibody coated to polystyrene particles (Dade Behring, Liederbach Germany, N Hs CRP, cat no OQIY G13; supplement reagent OUMU15).
CRP on the IMMAGE® Immunochemistry System from Beckman Coulter is measured by peak rate nephelometry with a laser diode at 670 nm. A polyclonal anti-CRP antibody is used (Beckman Coulter, cat no 447280).
The new hs-CRP on the IMMAGE® (called CRPH) is measured by a near infrared particle immunoassay (NIPIA) with a laser diode at 940 nm, using a polyclonal and monoclonal anti-CRP (goat and mouse) antibody coated to latex particles (Beckman Coulter, Inc. Fullerton, CA, US, cat no 474630).
CRP analysis on the Synchron LX®20 System, a routine clinical chemistry analyzer from Beckman Coulter, is based on immunoturbidimetry, using a polyclonal anti-CRP antibody (Beckman Coulter, Inc. Fullerton, CA, US, cat no 465131). In this study, values below 5 mg/L are reported as 4.999 mg/L.
Table 2.1 presents the assay characteristics of the different methods used.
Samples for imprecision studies were prepared from five serum pools in the range from 0.2 – 50 mg/L, and from two serum pools in the range from 100 – 350 mg/L as determined on the IMMAGE. Within-run precision was obtained by measuring one sample 20 times within a single run. Between-run precision was obtained by measuring each level on 20 consecutive days, based on a single calibration.

Table 2.1. Characteristics of the different CRP methods.

Analyzer	Method	Dilution	Assay range	Manufacturer's claimed	
				Measuring range	Detection limit
IMMULITE	hs-CRP	1:100	0.10 – 500	0.10 – 500	0.10
BNA	hs-CRP	1:20	0.18 – 11	0.18 – 1150	0.18
		1:100	0.88 – 55		
		1:400	3.5 – 220		
		1:2000	17.5 – 1100		
IMMAGE	hs-CRP	neat	0.20 – 60	0.20 – 1440	0.20
		1:6	0.20 – 360		
		1:24	0.20 – 1440		
IMMAGE	CRP	1:36	– 80	1.0 – 960	1.0
		1:216	> 80		
Synchron LX20	CRP	neat	5 – 200	5.0 – 488	5.0
		ORDAC[a]	150 – 488		

[a] ORDAC = Over Range Detection And Correction; when a sample is ORDAC-ed, the LX20 uses a smaller sample size for analysis. Values are given in mg/L.

Linearity studies were performed with two samples containing about 50 mg/L and 250 mg/L CRP. Using the manufacturer's diluent eleven dilutions of each sample were made and measured in five replicates.
The serum index measured on the Synchron LX20 was used to indicate the sample condition in terms of icterus, hemolysis or lipemia. The system monitors the absorbance at 340, 410, 470, 670 and 700 nanometers and solves a set of equations to determine the response for each index. The response is directly proportional to the sample condition. The lipemia index is indicated in different levels 1 – 10. Level 1 is normal, indicating absence of lipemia. Sample turbidity was also assessed by macroscopic examination.
To assess the agreement between serum and plasma for the hs-CRP IMMAGE method, serum, EDTA and heparin plasma samples were collected from 25 patients.

Statistics

hs-CRP distributions were skewed rightward and therefore their values are expressed as medians ± SD. Agreement between methods was assessed visually by the method of Bland and Altman[5] and by means of Deming regression analysis[6]. Furthermore, percentile values were estimated and compared. For determination of reference values, the 0.95 nonparametric interpercentile interval was calculated[7]. Gender differences in samples from blood donors were assessed by the Mann-Whitney U test, while median serum versus plasma comparisons were assessed by the Wilcoxon matched pairs signed-ranks test.

Results

Precision and linearity data

The hs-CRP IMMAGE method appeared to be linear over the whole measured CRP concentration range and comparable to results of BNA (Figure 2.1A-B). For five pools with mean concentrations of 0.23, 1.22, 2.18, 16.94 and 44.46 mg/L between run variations of respectively 4.9, 6.7, 2.7, 2.7 and 2.1 % and within run variations of respectively 5.9, 4.3, 3.5, 2.1 and 3.0% were found. So, the total imprecision for the hs-CRP IMMAGE method remained below 7%. For two additional samples with concentrations of 168 mg/L and 342 mg/L within run variations remained bellow 6.2% for all methods.

Agreement between methods

Figures 2.2A-F show Bland Altman plots for the different method comparisons. Deming regression analysis in the region from 0 – 10 mg/L (Table 2.2) suggests a slightly better agreement of the hs-CRP IMMAGE with BNA (r = 0.991) as compared to IMMULITE (r = 0.986).

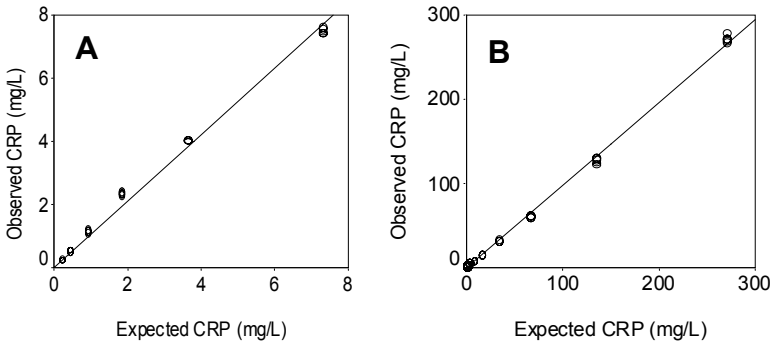

Figure 2.1. Linearity of CRPH IMMAGE® up to 10 mg/L an over the whole measured range.
Linearity results for hs-CRP IMMAGE (*A, B*) in the low (0 – 10 mg/L) and high (0 –300 mg/L) CRP concentration range. Dilution samples were run in five replicates. The mean values for each dilution point were plotted vs. expected values and linear regression was performed. Regression analysis results for the respectively low range were: y = 1.012 (SE 0.034) x + 0.201 (SE 0.118), r = 0.998, SEE 0.207; and for the high range: y = 0.989 (SE 0.012) x – 1.335 (SE 1.133), r = 0.999, SEE 3.204.

Table 2.2. Deming regression analysis of the hs-CRP IMMAGE method with other hs-CRP methods.

Analyzer	Slope ± SD	Intercept ± SD	SEE ($S_{y|x}$)	r
0 – 10 mg/L				
IMMAGE vs. BNA	1.10 ± 0.18	0.01 ± 0.17	0.39	0.991
IMMAGE vs. IMMULITE	1.37 ± 0.25	0.01 ± 0.18	0.54	0.986
IMMULITE vs. BNA	0.80 ± 0.11	-0.01 ± 0.10	0.22	0.989
10 – 300 mg/L				
IMMAGE vs. BNA	1.03 ± 0.14	0.05 ± 0.19	8.09	0.995
IMMAGE vs. IMMULITE	1.16 ± 0.27	0.01 ± 0.22	12.82	0.980
IMMULITE vs. BNA	0.89 ± 0.15	-0.06 ± 0.18	8.92	0.979

For the region from 0 - 10 mg/L, also percentile comparisons of the current CRP and new hs-CRP IMMAGE method to BNA were examined and are shown in the Figures 2.3A-B. The hs-CRP IMMAGE showed excellent agreement with BNA in all quartiles (Figure 2.3A), whereas the current CRP IMMAGE method showed good agreement only with hs-CRP concentrations above 75th percentile (Figure 2.3B). Samples determined on LX20**,** clear on macroscopic examination, showed no discrepancies with the high-sensitivity methods, whereas this was not the case for turbid samples (data not shown).

Between all hs-CRP methods compared large discrepancies were seen above 100 mg/L (Figure 2.1). Deming regression results are also presented in Table 2.2. Among the hs-CRP methods (Figure 2.2A-F) also for the whole tested range the best agreement was observed between hs-CRP BNA and hs-CRP IMMAGE. Comparison of the hs-CRP

methods with our routinely used CRP method (Synchron LX20) only resulted in a good correlation with the BNA (data not shown).

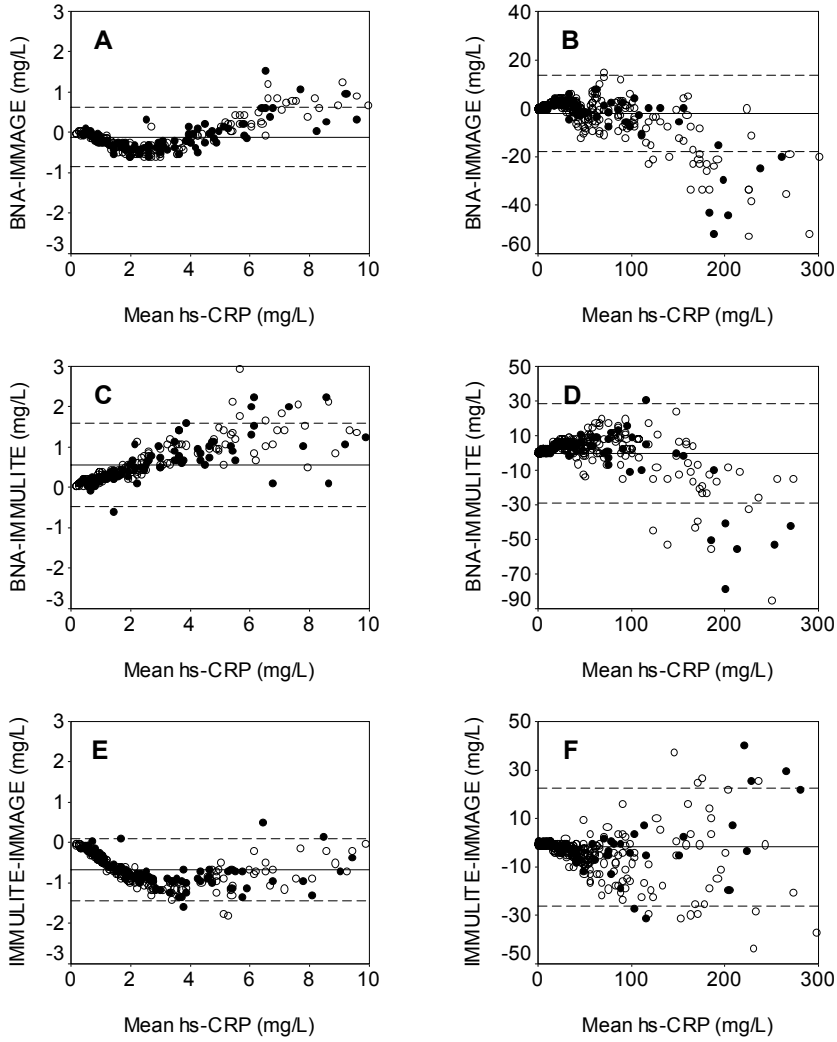

Figure 2.2 Method comparisons in 521 samples from patients with normal and increased CRP concentrations.

Bland-Altman analysis for 0 – 10 mg/L (A, C, E) and for 0 – 300 mg/L (B, D, F). The *solid line* indicates the mean difference between the methods, and the 95% confidence intervals for the difference are indicated by *dashed lines*. Mean bias and SD for Bland-Altman plots were –0.1 and 0.4 mg/L (A), 2.0 and 7.9 mg/L (B), 0.6 and 0.6 mg/L (C), 0.2 and 14.3 mg/L (D) –0.7 and 0.4 mg/L (E) and 1.8 and 12.1 mg/L(F).

Closed circles indicate samples showing turbidity assessed by macroscopic examination, whereas open circles indicate completely clear samples.

Serum versus plasma

Mean recoveries (± SD) of heparin- and EDTA-plasma related to serum concentrations were: 99.6 ± 3.6 % and 106.4 ± 11.2 % respectively. The results of the regression analysis were as follows: heparin plasma = 0.997*serum –0.037, r = 0.9998, SEE 0.360; and EDTA-plasma = 0.963*serum –0.725, r = 0.996, SEE 1.429. No significant differences were found when serum, heparin- and EDTA-plasma were compared for the hs-CRP IMMAGE method (serum *versus* heparin-plasma, p = 0.52; serum versus EDTA-plasma, p = 0.09).

Reference values

Table 2.3 shows patient characteristics and CRP concentrations measured by the hs-CRP IMMAGE in 291 blood donors. The median CRP concentration was 1.57 mg/L and the 0.95 interpercentile interval was 0.20 – 11.24 mg/L for all samples together (males 0.20 – 14.29 mg/L, females 0.20 – 9.84 mg/L). When values above 10 mg/L were omitted, the whole group median value was 1.42 mg/L and the 0.95 interpercentile interval 0.20 – 7.30 mg/L (males 0.20 – 5.40 mg/L, females 0.20 – 8.89 mg/L).

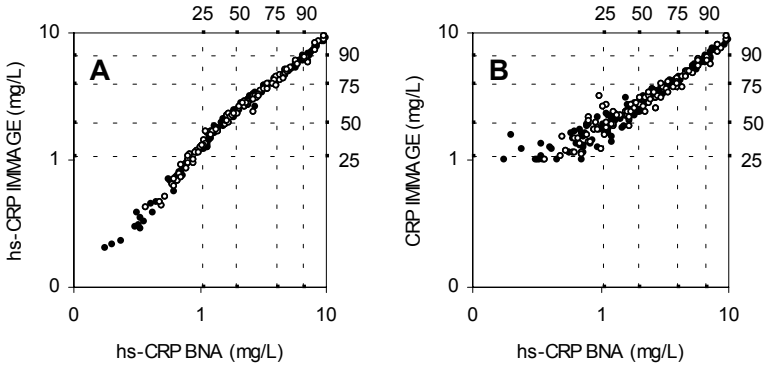

Figure 2.3. Accordance of the current CRP and the new CRPH IMMAGE® method.
Samples collected from 521 patients were analyzed by the current and new hs-CRP method, and the 25th, 50th, 75th and 90th percentiles were determined. A log scale was used for both the *ordinate* and *abscissa* in each panel. Results > 10 mg/L were excluded from each plot. (*A*), comparison of the results of the hs-CRP IMMAGE and hs-CRP BNA; (*B*), comparison of the results of CRP IMMAGE and hs-CRP BNA. Closed circles indicate samples showing turbidity assessed by macroscopic examination, whereas open circles indicate completely clear samples.

Table 2.3. CRPH IMMAGE and percentiles in blood donors.

Parameter	All	Male	Female
N[a]	291 (**282**)	177 (**170**)	114 (**112**)
Age, years			
Mean	48.3	48.6	47.8
SD	8.10	8.25	7.87
Median	49	50	47
Range	23 - 69	25 - 69	23 - 68
CRP, mg/L			
SD	2.94	3.20	2.48
Percentiles[a]			
2.5th	0.20 (**0.20**)	0.20 (**0.20**)	0.20 (**0.20**)
25th	0.67 (**0.65**)	0.64 (**0.62**)	0.83 (**0.80**)
50th(median)	1.57 (**1.42**)	1.25 (**1.20**)	1.86 (**1.82**)
75th	3.06 (**2.86**)	3.03 (**2.81**)	3.18 (**3.00**)
90th	4.98 (**4.62**)	4.89 (**4.30**)	5.87 (**5.62**)
95th	7.71 (**5.50**)	6.41 (**4.98**)	8.11 (**7.42**)
97.5th	11.24 (**7.30**)	14.29 (**5.40**)	9.84 (**8.89**)
99th	15.70 (**8.88**)	19.94 (**6.72**)	13.12 (**9.74**)

[a] Values between brackets represent hs-CRP values without samples higher then 10 mg/L.

Discussion

Precision and linearity

The new hs-CRP IMMAGE method was found linear in the concentration range of 0.2 – 10 mg/L, which was in agreement with the requirements for hs-CRP assays[3]. Furthermore, for risk stratification for cardiovascular, cerebrovascular, and peripheral vascular disease, the hs-CRP assay imprecision should be less then 10% at a concentration of 0.2 mg/L[3,5], which was also found for the new method. Given the relatively large within-subject variability of about 40%[5], analytical CVs of 10% or less should be adequate for both clinical and epidemiological studies.

Method comparison and interferences

In the low CRP range (0 – 10 mg/L) the new hs-CRP IMMAGE method showed good agreement with hs-CRP methods on BNA and IMMULITE, based on visual inspection of Bland-Altman plots (Figure 2.2A, C, E, G, I and K). In the concentration range from 0.2 – 10 mg/L the new IMMAGE hs-CRP method showed excellent agreement with BNA in all quartiles (Figure 2.3A), whereas the current IMMAGE CRP method showed good agreement only for concentrations above 75th percentile (Figure 2.3B). Roberts et al. performed percentile analysis to allow comparison with a recent study, where the current IMMAGE CRP method was included in the comparison[2]. For the percentile comparison, in our study, BNA was taken as a reference method, whereas Roberts *et al.* used the BN II N hs-CRP assay[2].
In our study, performed on 521 samples, for all hs-CRP methods good agreement was found for concentrations below 100 mg/L.

Above 100 mg/L large discrepancies were seen between the methods, whereas rather good agreement was observed only between LX20 and BNA. Roberts *et al*. found excellent agreement between the compared methods up to 50 mg/L in 50 patients[2]. Furthermore, in our study, above 100 mg/L, the scatter observed was more then 2SD, which could not be explained solely by proportional bias or imprecision data in the higher range, but is more likely due to assay or system related issues (Figures 2.2B, D, F). The scatter that was more then 2SD (above 100 mg/L), was observed for both the CRP IMMAGE (data not shown) and the new hs-CRP IMMAGE method when compared to BNA (reference method in our study).
Visual inspection of Bland-Altman plots revealed that sample turbidity did not interfere in the hs-CRP methods, as no systematic discrepancies were seen. With Synchron LX20, reliable CRP measurements could only be obtained in sera clear on macroscopic examination (data not shown).

Reference values

The median CRP concentration was 1.57 mg/L and the 0.95 interpercentile interval was 0.20 – 11.24 mg/L for all samples together (males 0.20 – 14.29 mg/L, females 0.20 – 9.84 mg/L). When values above 10 mg/L were omitted, the whole group median value remained higher then median values reported by others (0.58 – 1.13 mg/L)[2,4]. This is probably due to the already observed weak positive correlation between age and hs-CRP concentration (median age of healthy blood donors in our study was 49 years compared to 32 years reported in previous studies)[2]. It might also be due to eventually unreported episodes of cold within the prior 90 days[8]. The gender difference in hs-CRP was not found significant, neither before ($p = 0.15$) nor after omitting of the values ($p = 0.06$) higher then 10 mg/L, as is in accordance with previous studies[2,8].

Conclusion

In conclusion, evaluation of the hs-CRP method on the IMMAGE yielded good imprecision results and satisfying linearity as required for hs-CRP methods. The method correlated well with the other two compared hs-CRP methods in the range from 0.2 – 100 mg/L. However, above 100 mg/L poor correlations were found between all different methods, both hs-CRP and CRP. Therefore, the use of two different methods, a hs-CRP for risk stratification and a normal routine CRP method, seems still required.
As we are convinced that for the clinical laboratory it is most practical to use one CRP method for the complete measuring range, further investigation to improve agreement between the different methods also in the higher range is required.

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