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A new high-sensitive nephelometric method for assaying serum C-reactive protein based on phosphocholine interaction

Abstract

Background: The measurement of C-reactive protein (CRP) concentrations has been of interest as a classical marker of acute phase response; in addition, it has been of particular interest in cardiovascular risk stratification where high-sensitive measurements are necessary. Since CRP is able to bind phospholipids (mainly phosphocholine) in the presence of calcium ions, we explored the possibilities of developing a high-sensitive affordable nephelometric CRP assay based on diluted soy oil emulsions.

Methods: Serum (or heparinized plasma) was mixed with Intralipid 20% in Tris-calcium buffer (pH 7.5). After 12 min of incubation at 37°C, the CRP-phospholipid complexes were measured by nephelometry (840 nm) using a BN II nephelometer (Siemens). Results (n=97) were compared with those obtained using a typical immunoturbidimetric method (Roche).

Results: Imprecision of the functional nephelometric assay was evaluated using three human serum pools. Within-run coefficients of variation (CVs) for level 1, 2 and 3 were 6.1%, 4.7% and 4.5%, respectively, and between-run CVs were 17.6%, 18.8% and 11.3%, respectively. Good agreement was obtained between the functional nephelometric and the immunoturbidimetric CRP assay in a concentration range from 0.1 mg/L to 50 mg/L (r=0.884). A logit-log calibration curve was made between 0.056 mg/L and 1.785 mg/L. The limit of detection was 0.5 mg/L.

Conclusions: The functional nephelometric CRP assay allowed high-sensitive CRP determinations in serum and plasma. Since the assay is species independent, the described functional CRP assay could be used for veterinary purposes as well.

Keywords: C-reactive protein; nephelometric assay; phosphocholine.

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Introduction

C-reactive protein (CRP), the classical marker of acute phase response in humans and most animals, is an indicator of a variety of pathologic processes including infections, tissue damage, and chronic inflammatory diseases [1]. Serum CRP concentrations may rise within 24–48 h after the acute event and may increase up to a 1000-fold. When released in the circulation it is equally distributed in the vascular compartment without substantial tissue sequestration at sites of inflammation [2]. In addition, since the clearance of CRP is monoexponential and independent of the serum concentration or pathophysiological circumstances, measurement of CRP is a good marker of disease activity and it may contribute to the diagnosis and management of infectious diseases and other inflammatory conditions [2–4].

During the past decade, CRP experienced a revival in attention due to its possible role in the inflammatory pathogenesis of cardiovascular diseases [2, 5–7]. The CRP concentration is supposed to reflect the grade of vulnerability and instability of the atherosclerotic plaques, leading to rupture, thrombosis and thus, to occlusive arterial disease. Since atherosclerosis remains the major cause of death in developed countries and despite the successful fight against lipid associated risk factors, the role of inflammation in atherosclerosis has become more evident. High-sensitivity CRP is regarded as an excellent parameter to predict the grade of inflammation in relation to atherosclerosis and patients with intermediate risk of coronary heart disease. Multiple prospective studies in initially healthy subjects (excluding active inflammatory

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conditions) have shown an association between concentrations of CRP within the reference range (<5 mg/L) and future major cardiovascular events. This has led the Center for Disease Control and Prevention and the American Heart Association to publish a statement for health professionals addressing recommendations for the use of CRP measured by high-sensitive assays (hsCRP) in cardiovascular risk prediction, particularly among patients at intermediate risk of coronary heart disease [5, 8, 9].

Currently, most clinical laboratories predominantly rely on automated immunonephelometric and immunoturbidimetric assays to quantify CRP concentrations with detection limits of 0.3–5 mg/L, depending on the purpose of the CRP measurement, e.g., in acute inflammatory processes or for cardiovascular risk stratification [9, 10]. Several other techniques have been studied, using a wide variety of nanoparticles with different detection systems, e.g., selective ligands such as phosphocholine or o-phosphorylethanolamine and emerging techniques based on surface plasmon resonance and molecular imprinted polymers; these seemed quite promising due to their short reaction time and high sensitivity [10]. In addition, non-immunological methods are preferable in veterinary medicine, since immunoassays are not able to detect CRP from unrelated animal species due to the absence of common antigenic sites.

Calcium-mediated CRP binding to phosphocholine has been the subject of several studies, using different reagents such as synthetic globular polymers as scaffolds for the phosphocholine molecules, or soy oil emulsions [11–13]. Both in vitro and in vivo, CRP has been reported to produce agglutination ('creaming') of the intravenously administered lipid suspension Intralipid [14, 15]. Similarly, CRP also produced agglutination of isolated normal very low-density lipoproteins (VLDL) [16, 17]. Tugirimana et al. developed a manual turbidimetric CRP assay based on this agglutination with phosphocholine using Intralipid 20%, intended for use in Third World district hospitals [18]. However, this method had an analytical sensitivity of only ±7 mg/L, which is slightly above the reference range (5 mg/L).

In view of the clinical importance of hsCRP and the relatively high above described limit of detection (LOD), special attention was paid to develop a method with a good analytical sensitivity. Since particle-enhanced nephelometry has enabled measuring CRP concentrations as low as 10^{-9} mol/L [19], we explored the possibilities of developing an affordable functional method for assaying CRP in serum or plasma using soy oil emulsions containing phosphocholine (Intralipid 20%) as a natural and cheap nephelometric reagent.

Materials and methods

Study population

Ninety-seven randomly selected serum samples with varying CRP concentrations (mean=52.0 mg/L, range=0.7–171.6 mg/L) were obtained from the routine laboratory. Venous blood was collected, allowed to clot and centrifuged at 2000 *g* for 20 min. Subsequently, the samples were stored at 4°C for up to 1 week prior to testing.

Immunoturbidimetric assay

The latex particle enhanced immunoturbidimetric assay for CRP, regarded as a standard technique, was carried out using CRPL3 reagent (Roche, Mannheim, Germany), with a Cobas 8000 analyzer (Roche). The assay has been standardized using a commercial standard traceable to CRM 470 [20–22] (Roche).

Functional nephelometric CRP assay

Intralipid 20% (Fresenius Kabi, Uppsala, Sweden) is a fat emulsion for intravenous infusion containing, per liter, 200 mL of soybean oil, 12 mL of egg phospholipids, and 22 mL of glycerin, with the balance made up by water. Intralipid 20% is stable for at least 6 weeks at 37°C. Hydroxymethyl aminomethane (Tris) and calcium chloride were purchased from Sigma-Aldrich, sodium chloride was purchased from Merck (Darmstadt, Germany).

The functional nephelometric CRP assay was carried out using Intralipid 20% and Tris-calcium chloride buffer [Tris (hydroxymethyl aminomethane) 0.1 mol/L; calcium chloride 0.1 mol/L; pH was adjusted to 7.5 using hydrochloric acid 1 mol/L], on a BNII nephelometer (Siemens, Marburg, Germany) using a two-step protocol. Intralipid 20% was manually prediluted (1:700) with Tris-calcium chloride buffer.

During the first step, 30 µL of serum was mixed with 30 µL of 0.9% NaCl and 175 µL of Tris-calcium chloride buffer; followed by the addition of 50 µL of 0.9% NaCl and 50 µL of prediluted Intralipid 20% in a second step. The commonly used commercial diluent solutions containing phosphate-buffered saline should not be used for the functional CRP assay since they cause, in combination with the Tris-calcium chloride buffer, a calcium phosphate precipitate in the cuvette, which results in an interference. All serum samples were automatically prediluted (1:100) with 0.9% NaCl. CRP concentrations were measured nephelometrically after 12 min incubation, based on the absolute increase of the nephelometric signal over 12 min. A more detailed description of the applied instrument settings can be found in Table 1. The functional assay was calibrated using a commercial primary standard traceable to CRM 470 (Roche).

Affinity chromatography

In order to obtain CRP-depleted serum, affinity chromatography of pooled serum with a low CRP concentration (<0.6 mg/L) was carried out against phosphocholine containing gel (Thermo Fisher Scientific, Rockford, USA) [23]. A 5-mL column was prepared by packing

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Table 1 Instrument settings for the functional nephelometric CRP assay (BNII).

Parameter	Setting
Two-step protocol:	
Step 1:	
– 0.9% NaCl	30 µL
– Tris-calcium chloride buffer	175 µL
– Sample (serum/plasma)	30 µL
– Mixing time	default (0.0 s)
Step 2:	
– 0.9% NaCl	50 µL
– Prediluted Intralipid® 20%	50 µL
– Mixing time	Default (0.6 s)
Intralipid® 20% predilution	1/700 using Tris-calcium chloride buffer
Calibration	
– Start dilution	1/20 using 0.9% NaCl
– Dilution points	6
– Concentration range	0.056–1.785 mg/L
Start sample dilution	1/100 using 0.9% NaCl
Reaction time	12 min
Reaction temperature	37°C

the phosphocholine containing gel into a disposable column. The column was equilibrated by applying two column volumes of binding buffer (0.1 M Tris buffer, 0.1–0.2 M NaCl, 1–2 mM $CaCl_2$; pH 8). Subsequently, the pooled serum was added to the column, incubated for 1 h at room temperature and the CRP-depleted serum was collected for further research. The column was washed with five column volumes of binding buffer and the bound protein (purified CRP) was eluted by substituting EDTA for the Ca^{2+} in the buffer (elution buffer: 0.1 M Tris buffer, 0.1–0.2 M NaCl, 2 mM EDTA; pH 8). In addition, the CRP concentration of the supposed CRP-depleted serum was subsequently measured using the immunoturbidimetric assay on a Cobas 8000 analyzer and was not detected (0.0 mg/L, LOQ 0.6 mg/L).

Performance characteristics

The limit of blank (LOB) was calculated as the mean value +3 standard deviation (SD) for a blank sample (CRP-depleted serum). The lower LOD and the limit of quantification (LOQ) were calculated as the mean value +3SD and as the mean value +6 SD for a sample with a very low CRP concentration, respectively [24]. Linearity was evaluated in the concentration range between 1 and 300 mg/L [21]. Within-run (n=10) and between-run (n=8) imprecision were assessed using human serum pools with CRP concentrations ranging from 2.5 mg/L to 65.8 mg/L. Within-run analysis was performed in 1 run in one day and between-run on 4 consecutive days with two analyses each day.

Specificity

The purified CRP protein (obtained by affinity chromatography) was used to assess the specificity of the assay. In order to investigate the possible effects of confounding factors, serum calcium, cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, phospholipids, and total and direct bilirubin were measured in parallel using standard methods. Triglyceride, cholesterol, HDL-cholesterol, total and direct bilirubin, and serum calcium concentrations were measured for all serum samples using a Cobas 8000 analyzer (Roche).

Method comparison

Comparison was carried out between the immunoturbidimetric assay (CRPL3, Cobas 8000) and the functional nephelometric CRP assay (n=97) during 3 consecutive days.

Statistics

Data were analyzed using MedCalc® (MedCalc Software, Mariakerke, Belgium). Agreement between the functional nephelometric assay and the immunoturbidimetric assay was evaluated using Passing-Bablok regression analysis. Multiple regression analysis was used to investigate potential confounding factors; results with a $p<0.05$ were considered significant.

Results

Performance characteristics

A six-point calibration curve of the nephelometric signal (bits, measurement of the scattered light intensity in a fixed angle of 13–24°) and CRP concentration was constructed (Figure 1). A mean deviation of all curve points <10% was targeted, however <6% was overall easy to achieve. The LOB was 0.05 mg/L, based on the experiment with the CRP-depleted serum (a small negative mean bit value of –25 was observed). The LOD and the LOQ of the functional nephelometric CRP assay were 0.5 mg/L and 0.8 mg/L, respectively. The assay was found to be linear in the range between 0.1 mg/L and 300 mg/L. Within-run and between-run imprecision data are given in Table 2. The within-run coefficients of variation (CVs) ranged from 4.5% to 6.1% and between-run CV values were found between 11.3% and 18.8%.

Method comparison

We found a good agreement between the CRP values (mg/L) obtained by the immunoturbidimetric (X) and the functional nephelometric CRP (Y) methods in a concentration range from 0.1 mg/L to 50 mg/L: Y (CRP by functional

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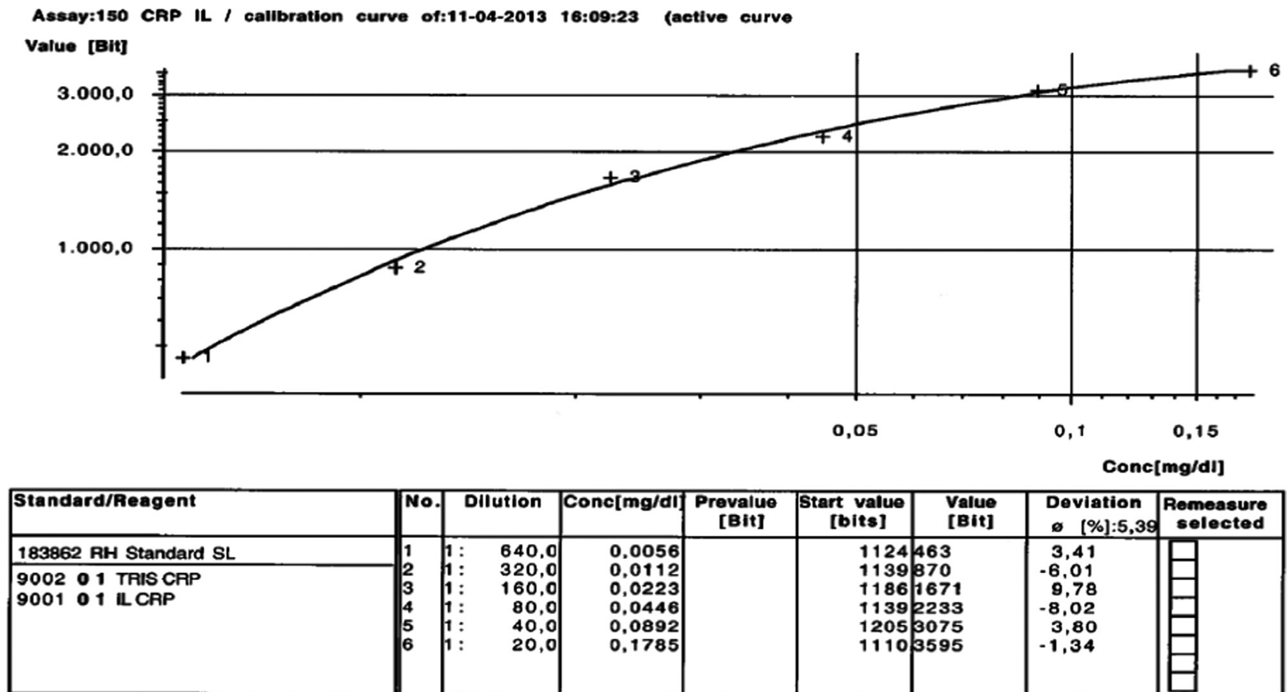

Standard/Reagent	No.	Dilution	Conc[mg/dl]	Prevalue [Bit]	Start value [bits]	Value [Bit]	Deviation ø [%]:5,39	Remeasure selected
183862 RH Standard SL	1	1 : 640,0	0,0056		1124	463	3,41	
9002 0 1 TRIS CRP	2	1 : 320,0	0,0112		1139	870	-6,01	
9001 0 1 IL CRP	3	1 : 160,0	0,0223		1186	1671	9,78	
	4	1 : 80,0	0,0446		1139	2233	-8,02	
	5	1 : 40,0	0,0892		1205	3075	3,80	
	6	1 : 20,0	0,1785		1110	3595	-1,34	

Figure 1 Calibration curve of the functional nephelometric CRP assay (BNII).
The start value (bit) represents the light scatter before the reaction takes place. This value is mainly determined by the light scatter properties of the Intralipid particles used. The next column (marked 'value bits') depicts the delta between the final reading and the initial reading. For samples containing small amounts of CRP (1:640 and 1:320 dilutions) the effect of CRP agglutination on the light scattering is small (and hence the bit value is lower than the initial start value).

assay; mg/L)=–3.212+1.017X (CRP by immunoturbidimetric assay; mg/L); r=0.884, p<0.0001. The 95% confidence intervals were [0.87–1.12] for the regression slope and [–5.25 to –1.63] for the intercept. Figure 2 shows the Passing-Bablok regression curve, while Figure 3 depicts the Bland-Altman difference plot between the functional and the immunoturbidimetric CRP methods.

In contrast, only a limited agreement was achieved between the CRP values obtained by both methods in a broad concentration range from 0.1 mg/L to 200 mg/L: Y (CRP by functional assay; mg/L)=–10.983+1.635X (CRP by immunoturbidimetric assay; mg/L); r=0.895, p<0.0001.

Specificity

Figure 4 represents the performance of the affinity chromatography method to obtain purified CRP or CRP-depleted serum. The electropherogram shows a single peak in the gamma region, which corresponded with the purified CRP. This illustrates that the selectivity of the protein binding to phospholine is extremely high.

No significant effect of serum phospholipids (up to 345 mg/dL), total cholesterol (up to 7.22 mmol/L), HDL-cholesterol (up to 2 mmol/L), triglycerides (up to 40 mmol/L) and calcium (up to 2.5 mmol/L) was observed

Table 2 Imprecision data for the functional nephelometric CRP assay (BNII).

CRP pools assayed by nephelometry, mg/L	Within-run CV (n=10)			Between-run CV (n=7)[a]		
	Mean, mg/L	SD, mg/L	CV, %	Mean, mg/L	SD, mg/L	CV, %
Level 1	2.5	0.1	4.5	–	–	–
Level 2	4.5	0.3	6.1	6.4	1.1	17.6
Level 3	21.6	1.0	4.7	26.1	4.9	18.8
Level 4	65.8	3.0	4.5	68.0	7.7	11.3

[a]Between-run imprecision was evaluated on four consecutive days with two analyses each day. However, there was a technical failure in the second run of day 4, therefore only seven results were available. (–, not available).

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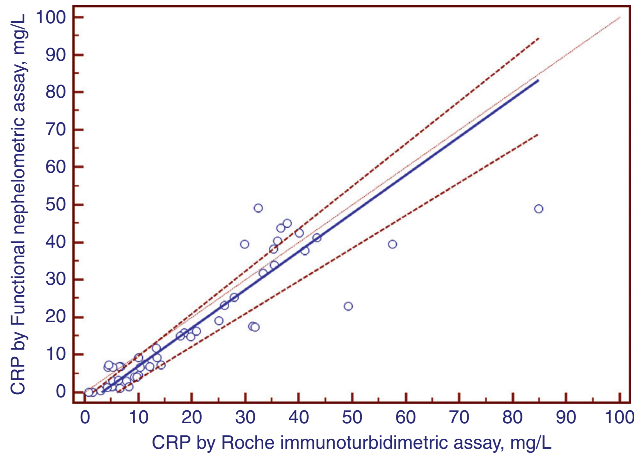

Figure 2 Method comparison functional nephelometric CRP assay (BNII) vs. immunoturbidimetric CRP assay (Cobas 8000) in a concentration range from 0.1 mg/L to 50 mg/L.
Passing-Bablok regression line with 95% CI (dashed lines) for 49 serum samples: Y (CRP by functional assay; mg/L)=−3.212+1.017X (CRP by immunoturbidimetric assay; mg/L); r=0.8837, p<0.0001. Dotted line: Y=X.

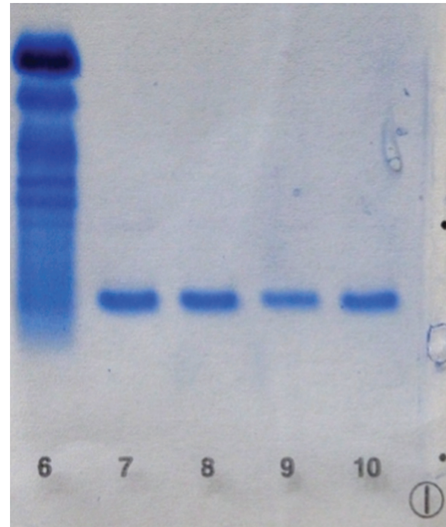

Figure 4 Agarose gel electrophoresis followed by Protur® staining (Analis, Namur, Belgium).
Lane 6 shows the diluted human serum pool (total protein concentration 1.5 g/L). Lanes 7–10 represent the purified CRP, showing a single peak in the γ region of the electropherogram.

on the correlation between the immunological and functional CRP assay.

Discussion

The assay we developed allows measurement of CRP in serum or heparin plasma using phosphocholine rich soy oil emulsions in the standard clinical range, in addition it can also be used as an assay for hsCRP measurements. When compared with the previously described manual functional method [18], the nephelometric assay has two advantages. At first, we were able to automate the manual method using nephelometry with a reaction time of only 12 min. Second, we were able to measure CRP as low as 0.5 mg/L; this in contrast with the manual method which was not able to detect CRP concentrations around the upper reference range (LOD 7 mg/L). The nephelometric approach offers the advantage of being much more sensitive than turbidimetric assays [19]. Furthermore, the BNII nephelometer has an inbuilt dilution station which allows a broad spectrum of sample dilutions to be obtained (from 1:2000 to 1:1) enabling reliable results over a very wide concentration range to be generated without altering the assay program. In addition, the default starting sample dilution factor used (1:100), makes the method insensitive towards sample matrix effects and other interferences. Finally, the variation in serum calcium concentration will be negligible compared with the final calcium concentration in the cuvette, in view of the large excess of calcium ions in the reagent. As the reaction principle of the functional nephelometric CRP assay is independent of the species, the presented assay is not limited to humans, but also offers interesting perspectives for assaying CRP in veterinary medicine [25].

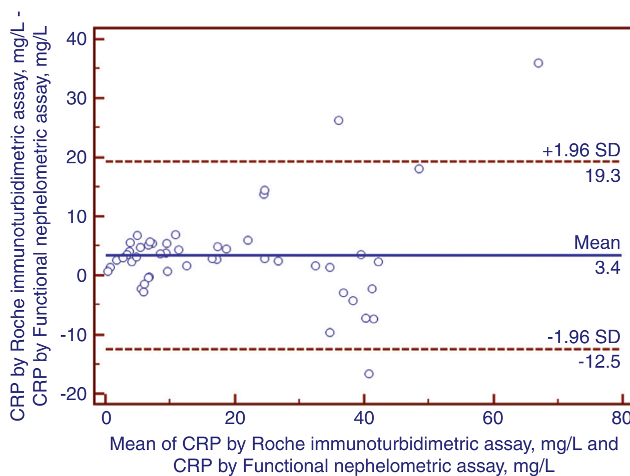

Figure 3 Bland-Altman plot expressing the difference between the functional nephelometric CRP assay (BNII) and the immunoturbidimetric CRP assay (Cobas 8000) against the mean of both measurements in a concentration range from 0.1 mg/L to 50 mg/L.
The solid line represents the mean difference; the dashed lines represent the 95% confidence intervals.

Three groups for cardiovascular risk prediction were proposed based on the following cut-off points for hsCRP concentrations: <1 mg/L for low risk, 1–3 mg/L for moderate risk and >3 mg/L for high risk [5]. A lower LOD

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of <1 mg/L and reproducibility data of <20% are prerequisites to obtain reliable results and to avoid potential misclassification of atherosclerotic risk. Most hsCRP assays have a lower LOD of <0.3 mg/L [5]. So, in contrast to the earlier described manual functional CRP assay [18], the present method is suited as a hsCRP assay. In addition, common turbidimetry is not sensitive enough to meet the required sensitivity for hsCRP measurements.

The assay conditions of the functional nephelometric CRP assay correspond with a final phosphocholine concentration in the cuvette of about 8 μmol/L, which is about four times the Kd value of CRP for phosphocholine [26]. In this way, impact of light scattering of the Intralipid® particles on the dynamic range of the test was kept to a minimum (±1100 bits, which accounts for ±10% of the total dynamic range of the detection system), while still keeping a comfortable phosphocholine binding capacity. In the clinically relevant CRP concentration range (0–100 mg/L), the CRP concentrations in the cuvette range between 0 and 1 μmol/L. The size distribution of the Intralipid lipid particles (diameter: 0.78±0.21 μm) [14] makes these particles ideally suited for functional turbidimetric analysis. Due to the multimeric structure of CRP, the natural lipid droplets act as scaffolds for the multivalent display of phosphocholine molecules in the newly developed functional methods.

The observed imprecison CV results (<6.5% for the within-run imprecision and <19% for the between-run imprecision) of the described functional method exceed the ones described for the immunoturbidimetric assay [27]. However, the functional nephelometric CRP assay fulfils the imprecision criteria based on biological variation (imprecision data of <21.1%) [28, 29]. The effect of sample turbidity using the functional nephelometric assay is very limited because of the high dilution factor of about 1:180 used in the assay. In addition, the wavelength (840 nm) chosen for nephelometric readings strongly limits optical interference by hemolysis and icterus. A good agreement was obtained between the functional nephelometric assay and the immunoturbidimetric assay in a CRP concentration range from 0.1 mg/L to 50 mg/L. However, a less good correlation was observed for a broader concentration range (0.1–200 mg/L), while the functional nephelometric assay has shown to be linear in a range of 0.1–300 mg/L. The small analytical bias (±3 mg/L) and the differences in the CRP results generated by the two assays can be partly explained by the genetic polymorphism of CRP [30] and the effect of phosphocholine-bound CRP isoforms. In addition, the functional CRP assay has the advantage of not being affected by epitopic variation. Furthermore, when using a 'standard platform', no instrument's reading system is capable to span a concentration range of four orders of magnitude. As a consequence, it is impossible to run clinical samples originating from an intensive care unit together with samples for hsCRP determination using one single assay.

The estimated cost of the described functional nephelometric assay would be as low as ±0.00001 €/test, vs. ±0.03 €/test for the manual functional assay [18] and ±1.0 €/test for an immunological test, this is because the assay requires inexpensive reagents, is technically simple and does not require the use of antibodies. In addition, the automated functional method allows a throughput of 112 tests/h. However, we are aware of the estimated cost of such a dedicated instrument as a BNII, but even when only functional CRP would be performed, running 35 samples/day would easily save ±800 €/year, which is already paying back the entire investment (in a poor country with limited resources). Furthermore, macroeconomic benefits (e.g., saving on antibiotics budgets, prevention of antibiotic resistance, etc) would be a major additional advantage.

This study demonstrates the potential of functional nephelometric CRP testing in human medicine. In addition, it offers interesting perspectives for applications in veterinary medicine [25].

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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