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Research paper

Validation of an immunoturbidimetric assay for assessment of C reactive protein in synovial fluid

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1. Introduction

The synovial fluid (SF) is a highly viscous solution localized in the articular joint that acts as a physiological lubricant reducing both friction and wear on the surfaces of articulating cartilages. It serves also as a physical barrier against mechanical stresses and has a nutritional function for the articular lining. The lubrication properties are mainly attributable to the high viscosity of the fluid, which is due to the presence of hyaluronic acid (sodium hyaluronate, HA), proteoglycan 4 and surface active phospholipids. These substances, accumulate in the synovial space by semipermeable synovial lining (Blewis et al., 2007). The concentration of HA in human SF ranges between 1 and 4 mg/mL in healthy individuals (Watterson and Esdaile, 2000; Mazzucco et al., 2004). Compared to all other body fluids SF composition is hence unique; consequently, both the handling of samples and the SF analysis have peculiar characteristics and are inherently challenging (Brannan and Jerrard, 2006; Block and Schimnich, 2013).

Several joint diseases are characterized by SF effusion and can be diagnosed by performing an arthrocentesis for assessing the cellular and chemical composition of SF (Margaretten et al., 2007; Pascual and Jovaní, 2005; Black et al., 2004). SF analysis usually consists of cell enumeration and differentiation, identification of crystals, along with measurement of a discrete number of biochemical parameters (Pascual and Jovaní, 2005).

The C reactive protein (CRP) was originally discovered in 1930 by Oswald Avery. The synthesis of CRP is exclusively hepatic and is triggered by interleukin–6 (IL6). The concentration of CRP may increase up to 1000-fold during an inflammatory response, reflecting enhanced synthesis by the hepatocytes. As regards its biochemical structure, the CRP is a pentraxin, i.e., a protein composed of five identical subunits, non-covalently associated around the central pore of the pentamer (Black et al., 2004).

For it can be associated with high morbidity and mortality rates, periprosthetic joint infection (PJI) can be regarded as one of the most serious complications following total joint arthroplasty. Moreover, it represents a remarkable economic burden on the health care system (Carpenter et al., 2011; Lavernia et al., 2006). Although a definite preoperative diagnosis of septic failure is necessary for an appropriate medical care, the diagnosis of PJI remains a considerable clinical challenge. Unfortunately, no 'gold standard' exists and no single test exhibits optimal accuracy for diagnosing this infection. Therefore, some infections may remain undiagnosed until surgery, thus favouring the deterioration of periprosthetic tissue and unfavourable clinical outcomes. In an effort to standardize the definition of PJI several orthopaedics and infectious diseases organizations have issued guidelines and expert opinions (Zmistowski et al., 2013). The Musculoskeletal Infection Society proposed a multi-criteria definition of PJI in 2011, which was then modified by a panel of experts during the International Consensus Meeting (ICM) of Philadelphia in 2013. According to ICM guidelines, the definition of PJI entails a combination of clinical features (sinus tract), SF analyses (leukocyte count, neutrophil percentage, fluid culture and leukocyte esterase), blood tests (erythrocyte sedimentation rate [ESR] and CRP), along with histological and microbiological tissue analysis. To establish a final diagnosis of PJI, either one of two major criteria or three of five minor criteria must be met.

CRP is currently measured in serum or plasma as a common and inexpensive test for the screening of PJI (Osmon et al., 2013). However, serum CRP lacks specificity for diagnosing localized infection since increased CRP levels in serum or plasma can be present in several non-infectious inflammatory disorders.

Recent studies have suggested that the measurement of CRP in SF might be regarded as a simple and cost-effective strategy for improving the diagnosis of PJI (Parvizi et al., 2006; Parvizi et al., 2012a,b). Wang et al have recently reported that the analysis of CRP in SF shows high sensitivity (0.92) and specificity (0.90) for diagnosing PJI (Wang et al., 2016). If these data will be confirmed by future studies, the analysis of

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CRP in SF could be introduced in routine clinical practice as a diagnostic criterion for ruling out the presence of PJI. Nevertheless, CRP is conventionally measured with a multitude of immunoassays that have only been validated using serum or plasma samples. According to the accreditation standard ISO 15189:2012, the laboratory performing tests on a biological sample matrix different from those designed by the manufacturers must validate the method in other biological fluids before introducing the test into clinical practice. Therefore, the present study aims at investigating the analytical performance of the OSR6147 Beckman Coulter immunoturbidimetric method for CRP quantification on Beckman Coulter AU480 clinical chemistry analyser in SF samples.

2. Materials and methods

2.1. Method description

The CRP OSR6147 assay is based on the principle that immune complexes in solution scatter the light according to their size, shape and concentration. This immunoturbidimetric assay hence uses goat anti-CRP antibodies, which form an immune-complex together with the CRP present in the test sample. The test is conducted using two liquid and ready to use reagents which are stable until expiration date when stored at 2–8 °C. Reagents are also stable for 90 days when stored in the refrigerated compartment of the analyser. The method is validated by the manufacturer for measuring CRP in serum and plasma samples which can be stored for a maximum of two months at 2–8 °C before analysis.

Some potential interfering substances have been reported by the manufacturer (Ashwood and Burtis, 1994). The criterion for establishing the lack of significant interference is the analyte recovery within 10% of the initial value. Bilirubin has no significant interference up to 400 mg/L, hemolysis has no significant interference up to 5000 mg/L, while lipemia has no significant interference up to 10,000 mg/L. However, the manufacturer declared that samples of patients with abnormal lipoprotein metabolism, such as those with cholecystitis or obstructive liver disease, may generate spuriously increased CRP values. In rare cases, oral contraceptives and immunoglobulin disorders (i.e., monoclonal or polyclonal gammopathy and Waldenström's macroglobulinemia) may be a source of significant interference.

The assay is calibrated with Serum Protein Multi-Calibrator ODR3021, which is traceable to the IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) standard CRM 470 (RPPHS). The Serum Protein Multi-Calibrator is a 6-level calibrator for serum protein assays, thus including CRP. According to the manufacturer's instructions, the calibration is supposed to be stable for 90 days. Recalibration is needed when the lot of the reagent changes, a shift in control values is observed, major preventative maintenance needs to be performed or a critical part of the analyser has been replaced. The quality control procedures are performed using two levels of an appropriate control material (ITA CONTROL SERUM ODC 0014 and ODC 0016 or Biorad Liquicheck, levels 1 and 2).

According to the manufacturer's specifications, the linearity of the assay is comprised between 5 and 300 mg/L both in serum and plasma. Samples with values exceeding the upper limit of linearity should be diluted with saline and either retested manually or repeated using an automatic rerun. Samples with extremely high CRP concentration (i.e., > 750 mg/L) may yield falsely low results without the appearance of the “Z” flags, which are usually associated with antigen excess in the test sample. In this case, the samples must be manually diluted with saline and retested. The total precision declared by the manufacturer is < 5%.

2.2. Method validation

This study was planned for validating the use of this immunoturbidimetric assay in SF samples and for comparing the values of CRP in paired serum and SF samples. The study was carried out according to the Clinical and Laboratory Standards Institute (CLSI) documents EP05-A3, EP15-A3, EP 17-A2, EP19-Ed2 (CLSI EP05-A3, 2014; CLSI EP15-A3, 2014; CLSI EP 17-A2, 2012; CLSI EP19-Ed2, 2015).

The LS samples included in the study were always collected in serum tubes (Vacuette Grainer Bio-One GmbH Austria) and transported at room temperature to the local clinical laboratory within 6 h from collection. All samples were immediately stored at −30 °C before analysis and analysed within 30 days from collection. The study was carried out in accordance with the declaration of Helsinki and approved by the local scientific committee of the Istituto Fiorentino Cura e Assistenza IFCA Firenze. The entire study was based on pre-existing samples anonymized soon after the routine testing had been completed.

2.2.1. Studies on hyaluronidase

A preliminary study was performed to define whether hyaluronidase (HY; Sigma Chemical Co., St. Louis, Mo) should be added to SF samples. In fact, it had been reported that the addition of this enzyme might be necessary for cell enumeration and differentiation in SF samples collected with K3EDTA tubes in order to prevent generating spurious results due to the high viscosity of SF (Sghezzi et al., 2016). A total number of 35 paired SF aliquots (with or without HY) were included in this part of the study. HY was used at a final concentration of 0.5 mg/mL, by dissolving 2.5 mg of HY in 5 mL of 0.1 mol/L phosphate buffered saline PBS (pH 7.4). The pre-treatment of SF samples consisted of mixing 20 µL of HY solution with 1 mL of SF, followed by incubation at room temperature for 5 min. An equal amount of PBS (i.e., 20 µL) was added to the paired aliquots designed as HY-free. The samples were then centrifuged at 3000 rpm for 15 min before the analysis; the difference of CRP values between HY-treated and HY-free aliquots was assessed with Bland-Altman regression.

2.2.2. Imprecision studies

The imprecision of the immunoturbidimetric assay was assessed according to the CLSI EP05-A3 protocol. A pool of SF samples with low CRP concentration (< 0.5 mg/L) was prepared by mixing specimens with undetectable CRP values. HY was then added to the pool, followed by centrifugation and CRP measurement. This pool, whose final CRP value was < 0.1 mg/L, was then used for preparing four additional samples by adding increasing volumes of CRP calibrator, thus yielding final CRP concentrations of 5, 11, 57 and 100 mg/L, respectively. These four samples were then tested in duplicate for 20 non-consecutive days (inter-assay imprecision) along with two levels of control material using the same lot of reagents and calibration.

2.2.3. Trueness study

Two reference SF samples with different concentrations of CRP were prepared as follows. Increasing volumes of CRP calibrator were added to the same SF pool used for the imprecision studies to produce two titrated solutions with final CRP concentration of 59 and 5.6 mg/L, respectively. Both samples were then tested in five replicates per day for five consecutive days using the same lot of reagents and calibration.

2.2.4. Dynamic range

The dynamic range, limit of blank (LOB), limit of detection (LOD) and limit of quantitation (LOQ) were estimated according to the CLSI EP 17-A2 standard. Two different lots of reagents were used for analysing four SF samples with undetectable values of CRP and four additional SF specimens with low CRP concentration (i.e., between 0.1 and 0.3 mg/L). A total number of 60 replicate measures were performed on both samples for each reagent lot. The LOB was calculated according to the CLSI EP 17-A2 standard, as follows: $LoB = M_B + cpSD_B$, where M_B is the mean of blank results in the dataset and SD_B, is the standard deviation of blank results in dataset, cp is the fixed coefficient $1645 / 1 - (1 / 4(B - K))$, B is the total number of blank results in dataset and K is number of blank samples. LOD was calculated as follows:

LOD = LOB + cpSD$_L$ where SD$_L$ is the pooled standard deviation of the low-level samples. LOQ was calculated according to the Eurakem Guide (Magnusson and Örnemark, 2014), as follows: LoQ = M$_B$ + 10 SD$_B$.

2.2.5. Linearity

The linearity of this CRP immunoturbidimetric assay was assessed using seven SF samples obtained by adding increasing amounts of CRP calibrator to a SF pool with undetectable CRP concentration, thus producing a range of CRP concentration comprised between 5 and 240 mg/L. The linearity was then estimated using Pearson's correlation and the evaluation by fitting 2nd or 3rd order equations by regression analysis showing in the latter an R^2 = 0,9999. The analyte recovery was also calculated using a linearity function.

2.2.6. Stability

The stability of CRP in SF was assessed using a sample with a concentration of 50 mg/L, which was tested in duplicate at the baseline and 2, 4 and 24 h thereafter. Results were finally expressed as coefficient of variation (CV).

2.2.7. Carryover

The carryover was assessed by analysing in triplicate a SF sample ("H") with high CRP value (240 mg/L) followed by an analysis in triplicate of another sample ("B") with low CRP concentration (< 0.2 mg/L). The carryover was then calculated using the conventional formula [[B1 − B3] / [H3 − B3]] × 100.

2.3. Statistical analysis

The statistical analysis was performed using MetComp ver. 1.0 (Matteo Vidali and GdS SIBioC Statistics for Laboratory, Novara, Italy).

3. Results

Although no statistically significant difference of CRP values could be observed between HY-treated and HY-free SF samples (mean bias, −1.16 mg/L; 95% confidence interval [95% CI], −2.93 to 0.61 mg/L) (Fig. 1), the samples treated with HY were better aspirated by the sample probe of the analyser. Moreover, a lower rate of undetectable values was observed. Therefore, the SF samples used in the following part of this study were all treated with HY. The inter-assay imprecision of the assay was comprised between 1.9 and 3.3% (Table 1).

Table 1
Imprecision studies of C reactive protein in synovial fluid samples.

Samples	Mean ± SD	CV
Sample 1 (mg/L)	104.8 ± 2.4	2.3%
Sample 2 (mg/L)	57.2 ± 1.7	1.9%
Sample 3 (mg/L)	11.0 ± 0.3	3.1%
Sample 4 (mg/L)	5.1 ± 0.2	3.3%

CV, coefficient of variation; SD, standard deviation.

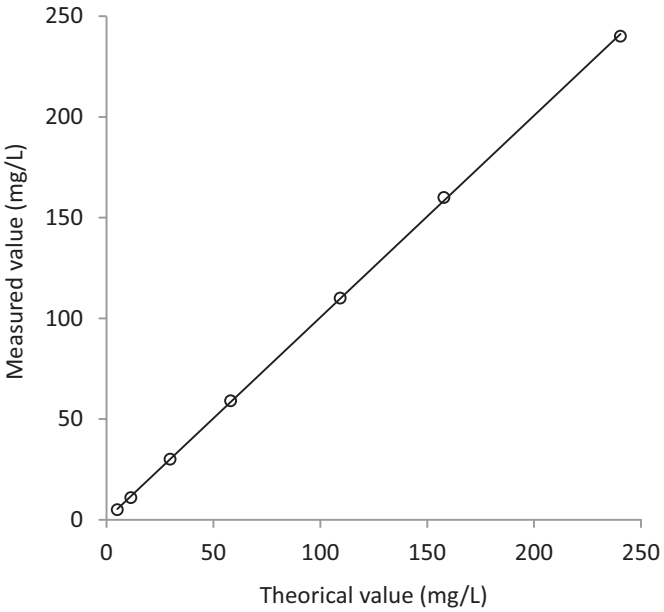

Fig. 2. Linearity of C reactive protein in synovial fluid samples.

The trueness of the method was also comprised between 1.4 and 3.9% for the two SF samples with CPR values of 59 and 5.6 mg/L, respectively. The estimated LoB, LOD and LOQ of CRP in SF were 0.65 mg/L, 1.09 mg/L and 2.9 mg/L. The linearity and recovery of the assay were excellent in a range of SF CRP concentration comprised

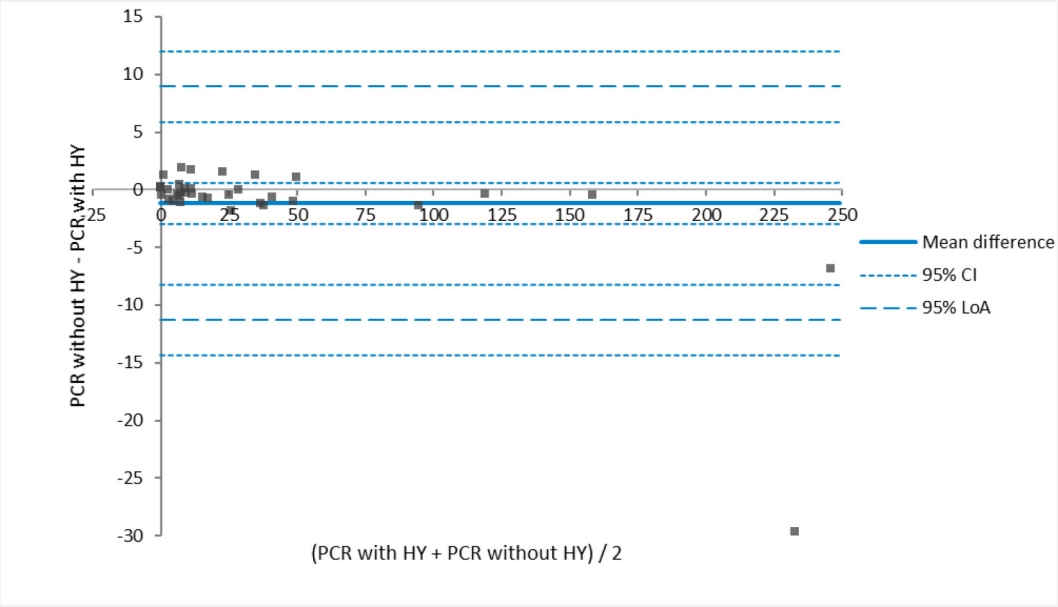

Fig. 1. Bland-Altman plot: difference of CRP values between HY-treated and HY-free.

between 5 and 240 mg/L (r = 1.00) (Fig. 2). The 24-hour stability of CRP in SF was also optimal, as confirmed by a total CV of 1.64%. Carryover was negligible (i.e., < 0.1%).

3.1. Limitations of the study

The limitations of this study can be summarised as follows: first, we used Serum Protein Calibrator to perform the validation of a different matrix (SF); secondly, in the accuracy studies we used only one pool of patients samples instead of at least two so as to avoid commutability problems and perform in duplicate the verification; and finally, we used CRP calibrator to evaluate linearity instead of employing patients samples or different pooled samples.

4. Discussion

The diagnosis of PJI remains challenging and often frustrating for both clinicians and patients. Although new biomarkers and innovative analytical applications of existing tests are continuously investigated (Parvizi et al., 2012a; Sousa et al., 2017; Suda et al., 2017), the current diagnostic armamentarium for diagnosing PJI is not characterized by high diagnostic accuracy in terms of both sensitivity and specificity. The assessment of CRP in serum or plasma has poor specificity for PJI, while a variable number of false negative results (i.e., up to 7%) are also encountered in patients with these conditions. Parvizi et al. have recently concluded that the diagnostic specificity of CRP could be considerably enhanced when this biomarker is measured in SF compared to the serum or plasma assessment (Parvizi et al., 2012a). However, before the CRP measurement in SF samples is introduced into clinical practice, or even becomes a new diagnostic paradigm, the current immunoassays will need extensive validation in this nonstandard body fluid (Lippi and Plebani, 2017).

Taken together, the results of our study show that the Beckman Coulter immunoturbidimetric assay applied on the Beckman Coulter AU480 clinical chemistry analyser may be also suitable for accurate quantification of CRP in joint effusions other than in serum and plasma, provided that some important requirements are met. Importantly, pretreatment of SF samples with HY seems necessary to optimize CRP assessment as previously described for SF cell counting (Buoro et al., 2017). When this provision is followed, the findings of our investigation confirm that LOB, LOD, LOQ, imprecision, trueness, linearity and carryover are suitable for this clinical purpose and are globally comparable to those claimed by the manufacturer for serum or plasma assessment. This evidence paves the way to further clinical studies aimed at clinically validating the measurement of CPR levels in SF for diagnosis and monitoring of patients with PJI and, predictably, in other joint inflammatory diseases. Therefore, our study will hopefully lay the groundwork to future investigations evaluating whether the CRP may be locally secreted in the SF or accumulate in joint effusion, thus representing a considerable step forward for both the diagnosis and treatment of joint inflammatory and the infectious disorders.

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