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A preliminary study of paraoxonase-1 in infected patients with an indwelling central venous catheter

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ABSTRACT

Objectives: Identification of biochemical markers to diagnose bloodstream infections in patients with a central venous catheter (CVC) inserted is an active research pursuit. Paraoxonase-1 (PON1) is an enzyme participating in the innate immune system protecting against toxic substances and infectious agents. We investigated the relationships between serum PON1 alterations and the characteristics of infection in a group of patients with a CVC implant.

Methods: Patients (n=114) who had had an inserted CVC removed because of infection or because the usefulness was at an end, and 407 healthy volunteers were recruited. In all participants we measured serum PON1 lactonase and paraoxonase activities, PON1 concentration and genetic polymorphisms, together with levels of the chemokine (C-C motif) ligand 2 (CCL2), procalcitonin and C-reactive protein (CRP).

Results: Patients with an acute concomitant infection (ACI) had higher CCL2, CRP and procalcitonin concentrations than the control group, together with lower paraoxonase and lactonase activities and specific activities. The areas under the curve of the receiver operating characteristic plots for paraoxonase and lactonase specific activities in the discrimination between patients with or without and ACI were 0.81 (0.73 – 0.89) and 0.81 (0.71 – 0.89), respectively, indicating the high diagnostic accuracy of these parameters.

Conclusion: This preliminary study suggests that the measurement of PON1 may be useful as a tool for the diagnosis of ACI in patients with an indwelling CVC.

Key words: Biomarkers; Catheters; Infection; Paraoxonase-1

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List of abbreviations: ACI, acute concomitant infection; AUROC, areas under the curve of receiver operating characteristic; CCL2, chemokine (C-C motif) ligand 2; CVC, central venous catheter; CRI, catheter-related infection; CRP, C-reactive protein; DTNB: 5,5'-dithio-bis-2-nitrobenzoic acid; EDTA, ethylene diamine tetraacetate; ELISA, enzyme-linked immunosorbent assay; HICPAC-CDC, Healthcare Infection Control Practices Advisory Committee of the Center for Disease Control and Prevention; ICU, intensive care unit; PON, paraoxonase; ROC, receiver operating characteristic; SPSS, Statistical Package for Social Sciences; TBBL, 5-thiobutyl butyrolactone.

1. Introduction

The use of central venous catheters (CVC) is ubiquitous in hospital care worldwide. These devices provide vascular access for the extraction of blood samples for analyses, infusion of intravenous drugs, parenteral nutrition, access for hemodialysis, and for hemodynamic monitoring. However, their use carries the risk of bloodstream infection, which is associated with increased morbidity, mortality and healthcare costs [1]. The conventional approach to the management of such infections involves decision-making regarding the removal of the indwelling catheter and the implementation of intravenous antibiotic therapy. Unfortunately, diagnosis of bloodstream infection is often complicated by symptoms such as fever, chills and hypotension, which are non-specific [2]. Therefore, to identify biochemical markers able to diagnose bloodstream infections accurately in patients with a CVC inserted is imperative, and constitutes an active line of research. Several studies have proposed C-reactive protein (CRP) or procalcitonin as useful markers of sepsis. However, their utility varies depending on the clinical setting, and this is still an unresolved issue [3].

The paraoxonases (PON) are a group of three lactonases (PON1, PON2, and PON3) ubiquitously expressed in human tissues, with antioxidant and anti-inflammatory properties. While PON2 is exclusively intracellular, PON1 and PON3 are also found in the circulation bound to high-density lipoproteins. PON1 degrades oxidized lipids in low-density lipoproteins and inhibits the synthesis of the chemokine (C-C motif) ligand 2 (CCL2), a pro-inflammatory molecule that attracts monocytes to the inflammatory sites and induces their differentiation to macrophages [4]. Schweikert et al. [5] suggested that PON2 and PON3 are an important part of our innate defense system against, for example, *Pseudomonas aeruginosa*. Moreover, PON2-deficient mice have a higher sensitivity to bacterial infection than wild-type mice [6,7]. Several lines of evidence suggest that PON1 participates in the protection conferred by high-density lipoproteins against different infectious agents, including bacteria [8,9] and viruses [10,11]. Overall, these results indicate that the PON family of proteins can be considered part of the innate immunity system [12].

Our study sought to characterize the alterations of PON1 levels in the circulation in patients with an implanted CVC, to relate them to their clinical and biochemical characteristics, and to investigate the potential utility of these parameters as biomarkers for the diagnosis of infection.

2. Materials and methods

2.1 Ethics approval

The study was approved by the Ethics Committee (Institutional Review Board) of the *Hospital Universitari de Sant Joan*. All the participants provided written informed consent to participation in the study on the understanding that anonymity of data was guaranteed.

2.2 Participants

We prospectively recruited, between March 2011 and June 2013, a total of 114 patients who had had an indwelling CVC removed because of infection, or because it was no longer needed according to the criterion of the attending physician. Exclusion criteria were: <18 years of age, severe alcoholism, psychiatric diseases, or liver impairment. Our protocol for management of an indwelling CVC is based on the recommendations of the Healthcare Infection Control Practices Advisory Committee of the Center for Disease Control and Prevention (HICPAC-CDC, 2002) [13]. At the time of catheter removal, a blood sample was obtained for biochemical and genetic analyses, and the catheter tip was cultured for microbiological analyses. The participants' medical records were reviewed and pertinent demographic data, comorbidities, bacteriologic and therapeutic data were recorded. Data on the patients' local and general infection-related clinical manifestations were also collected, as well as the presence of other acute or chronic infections. The type of treatment received in the 24h before the present study and the appearance of neoplasia after a 6 months follow-up, were recorded. The McCabe classification [14] and the Charlson comorbidity index [15] were recorded in all patients. Of the participants, 38 (33.3%) were hospitalized for surgery-related reasons and 22 (19.3%) for an infectious disease. The location of the catheter tip was brachial in 51 patients (44.7%), subclavian in 51 (44.7%), jugular in 9 (7.9%), and femoral in 3 (2.6%).

Patients with an acute concomitant infection (ACI) were those suffering from an infection (abdominal abscess, pneumonia, etc.) that was not related to an infected catheter. Twenty-two (19.3%) patients had an ACI without a catheter-related infection (CRI), 9 (7.9%) patients had a CRI without an ACI, and 14 (12.3%) patients had both infections simultaneously.

The control group consisted of 407 healthy volunteers who participated in an epidemiological study conducted in our geographical area, the details of which have been previously reported [16]. These subjects had no clinical or biochemical evidence of renal insufficiency, liver disease, neoplasia or neurological disorders.

2.3 Biochemical analyses

The physiological substrate, or substrates, for PON1 have not, as yet, been identified. Since PON1 has lactonase and esterase activities [4], we decided, in the present study, to measure the catalytic activity of PON1 using two different substrates: 5-thiobutyl butyrolactone (TBBL, a synthetic lactone) and paraoxon (an ester), as previously described [17]. Briefly, TBBLase activity was measured in an assay reagent containing 1mM $CaCl_2$, 0.25 mM TBBL and 0.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.05 mM Tris-HCl buffer, pH = 8.0. The change in absorbance was monitored at 412 nm. Activities were expressed as U/L (1 U = 1 mmol of TBBL hydrolyzed per minute). Serum PON1 paraoxonase activity was determined as the rate of hydrolysis of paraoxon at 410 nm and 37ºC in a 0.05 mM glycine buffer, pH 10.5 with 1 mM $CaCl_2$ [16]. Activities were expressed as U/L (1 U = 1 μmol of paraoxon hydrolyzed per minute). Serum PON1 concentrations were determined by an in-house enzyme-linked immunosorbent assay (ELISA) with a rabbit polyclonal antibody generated against the synthetic peptide CRNHQSSYQTRLNALREVQ which is a sequence specific for mature PON1 [18]. PON1 specific activities were calculated as the ratio between the activity and the corresponding concentration. The serum concentration of C-reactive protein (CRP) was measured using a high sensitivity method (Horiba ABX, Montpellier, France). The serum concentration of procalcitonin and the EDTA-plasma concentration of CCL2 were measured by ELISA (Biovendor, Brno, Czech Republic, and Prepotech, London, UK, respectively).

2.4 PON1 genotyping

Serum PON1 paraoxonase activity is strongly determined by the enzyme genotype [17]. Several polymorphisms in the promoter and the coding regions of the *PON1* gene have been described and, in the present study, we chose to analyze the polymorphisms Arg/Gln at position 192 (*PON1*$_{192}$, with two alleles termed Q and R), and the polymorphism Leu/Met at position 55 (*PON1*$_{55}$, with two alleles termed L and M). *PON1*$_{192}$ is strongly associated with the enzyme's activity and *PON1*$_{55}$ is a surrogate of the *PON1*$_{-108}$ promoter polymorphism [4]. For polymorphism analyses, genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set, Gentra Systems Inc., Minneapolis, MN, USA), and the chosen polymorphisms were analyzed by the Iplex Gold MassArray^{TM} method (Sequenom Inc., San Diego, CA, USA).

2.5 Microbiology analyses

Catheter tips were cultured by the semi-quantitative method of Maki et al [19]. Patients were classified according to clinical and microbiological criteria [20,21] as follows: “uninfected”, when there was no evidence of infection and catheter cultures were negative (CRI = 0); “localized catheter colonization”, when a significant growth of a microorganism (>15 colony-forming units) from the catheter tip was positive but without clinical evidence of infection (CRI = 1); “exit site infection” when there was erythema or induration within 2 cm of the catheter exit site (CRI = 2); “catheter-related bloodstream infection”, when catheter culture and blood culture tested positive for the same microorganism (CRI = 3). Subsequently, the identification and susceptibility testing of the culture isolate was performed by automated microdilution (MicroScan WalkAway®, Siemens Healthcare, Erlangen, Germany) and/or disk diffusion method and complementary biochemical tests, depending on the type of microorganism.

2.6 Statistical analyses

All calculations were performed with the SPSS 22.0 statistical package (SPSS Inc., Chicago, IL, USA). Differences between two groups were assessed with the Mann-Whitney *U* test, since most of the studied variables had non-parametric distributions. Differences between more than two groups were analyzed by the Kruskal-Wallis test. We fitted two binary logistic regression analyses to identify those variables independently associated with the presence of an ACI. One of the models included paraoxonase specific activity, and the other included TBBLase specific activity among the selected independent variables. Qualitative data were analyzed with the χ^2 test. Results are shown as medians and 95%CI. Spearman correlation coefficient was used to evaluate the degree of association between variables. The diagnostic accuracy of the investigated biochemical variables was assessed by receiver operating characteristic (ROC) curves [22].

3. Results

3.1 Clinical and demographic characteristics

Surgical intervention had taken place more frequently in patients with an infected catheter than in those patients without catheter infection. They also had a higher frequency of ACI, and were treated more frequently with antibiotics and/or parenteral nutrition. Brachial catheters had been implanted more often in patients who

did not develop a catheter infection. We did not observe any significant difference in any of the other studied variables, including McCabe and Charlson indices (Table 1).

3.2 Changes in the levels of infection/inflammation biomarkers and in PON1-related variables

Patients with a CVC had higher CCl2, CRP and procalcitonin concentrations than the control group, and lower paraoxonase and TBBLase activities. There were no significant differences in PON1 concentrations. Consequently, when we calculated the paraoxonase and TBBLase specific activities (i.e. the enzyme activities per gram of PON1 protein), we observed significant decreases in both parameters (Table 2). Differences were more marked in the patients with a higher CRI (Table S1. Supplementary material).

Patients were also separated according to whether or not they received antibiotic treatment. We observed statistically significant differences for PON1 concentration and paraoxonase specific activity (Table S2. Supplementary material). We did not observe any significant differences in PON1-related variables in patients with an ACI, when segregated according to the antibiotic treatment received (Table S3. Supplementary material). We did not observe any significant differences between patients and controls with respect to genotype frequencies of the $PON1_{192}$ and $PON1_{55}$ gene polymorphisms (Table S4. Supplementary material). When paraoxonase and TBBLase activities were segregated according to the $PON1_{192}$ isoforms, we observed that the average percentage decrease was higher in patients carrying the R allele (Table S5. Supplementary material). There were significant inverse correlations between several PON1-related parameters and procalcitonin and CRP concentrations (Fig. 1).

When the patients were segregated according to whether or not they had a CVC infection, we observed that patients with infected catheters had higher CCL2 and CRP, and lower paraoxonase and TBBLase activities and specific activities (Fig. 2). In addition, when the patients were segregated according to whether or not they had an ACI, we observed that patients having a concomitant infection had higher CCL2, CRP, procalcitonin, and PON1 concentrations, and lower paraoxonase and TBBLase activities and specific activities (Fig. 3).

3.3 Diagnostic accuracy of the infection/inflammation markers and PON1-related variables

When comparing the diagnostic accuracy of CRP, procalcitonin, CCL2 and the PON1-related variables in discriminating between patients with and those without an

ACI, we found that the AUROC mean values were higher for TBBLase and paraoxonase specific activities than for the classical biochemical markers of infection or inflammation; albeit an overlapping of the 95% CI was observed for the CCL2, CRP and procalcitonin variables (Figure 4A and Table 3). Conversely, CRP showed the best performance in discriminating patients with a catheter infection; again with overlapping of the other parameters (Figure 4B and Table 3). In addition, binary logistic regression analyses showed that both TBBLase and paraoxonase specific activities (and not CRP, procalcitonin or CCL2) were independently associated with the presence of ACI (Table 4).

4. Discussion

The present study showed a significant decrease in serum PON1 activity in patients with an indwelling CVC. This decrease was observed in the measured TBBLase (lactonase) and the paraoxonase (esterase) activities of this enzyme. PON1 hydrolyzes a broad range of substrates including esters, lactones, lipid peroxides, and estrogen esters [4]. This lack of substrate specificity is probably due to PON1 being an ancestral enzyme that appeared very early-on in evolution; it is present in many organisms ranging from invertebrates to mammals. Directed evolution studies, together with structure-function studies, established the primordial function of PON1 as that of a lactonase [23] which subsequently evolved new substrate specificities. Accumulating evidence indicates that the main function of PON1 is to degrade lipid peroxides and quorum-sensing lactones and, hence, plays an important role in the innate defense system against infection and oxidative stress [24]. Our results show that the decrease in PON1 activity in patients with an implanted CVC is not substrate-specific and involves lactonase as well as esterase activities. In addition, serum PON1 concentration did not decrease but remained unchanged, or even increased, in some subgroups of our patients. This implies that the decrease in activity was due to enzyme inactivation and, as a consequence, PON1 specific activities were also decreased. The inactivation of serum PON1 activity may be explained by several factors that are not mutually exclusive. Firstly, our patients had a high incidence of diseases associated with oxidative stress (diabetes mellitus, pulmonary disease, ischemic heart disease, dyslipidemia, cancer). PON1 degrades lipid peroxides, but the PON1 active site for their hydrolysis requires a free sulfhydryl group at cysteine 284, with lipid peroxides reacting covalently with this site leading to enzyme inactivation [25]. This implies that every PON1 molecule degrading an oxidized lipid becomes inactivated; the consequence being that the overall enzyme activity is decreased. Secondly, some bacteria can inactivate PON1 activity.

Quorum-sensing lactones secreted by Gram-negative bacteria mediate intracellular Ca^{2+}elevation in host cells, which causes rapid inactivation and degradation of PON1 [5]. Thirdly, some treatments have an inhibitory action on PON1 activity. These include digoxin, metoprolol, verapamil, diltiazem, amiodarone and methylprednisolone. In addition, antibiotics such as teicoplanin, rifampin, tobramycin, ceftriaxone, cefuroxime, ceftazidime, amikacin, ornidazole have been reported to inhibit the enzyme [26,27].

We did not observe any significant differences in *PON1* polymorphisms between patients with a CVC and the control group. However, we found that the average percentage decreases of paraoxonase as well as TBBLase activities were higher in patients carrying the R allele. This finding is of considerable note since this allele has been associated with several metabolic diseases, albeit under continuing scientific debate [28].

In daily practice, patients are hospitalized for various pathologies requiring the use of an indwelling CVC for the administration of life-preserving fluids including antibiotics, parenteral nutrition, whole blood, plasma, dextrose and saline. When fever occurs, it is difficult to ascertain if the source is the central catheter or a complication of the underlying disease. To find reliable biomarkers to discriminate between these etiologies is relevant from the clinical point of view because withdrawal of the CVC and insertion of a replacement is quite invasive and not exempt from risks such as pneumothorax or hemothorax. The most important conclusion of the present study is that TBBLase and paraoxonase specific activities have a good diagnostic accuracy in the diagnosis of ACI in patients with an indwelling CVC. The results were slightly better for TBBLase. Procalcitonin is a relatively good marker for invasive bacterial infections, while CRP is more non-specific and can be elevated in most inflammatory, neoplastic, and infective conditions [29]. These markers have been observed to vary considerably in different clinical settings. Generally, procalcitonin has shown a better performance than CRP in the diagnosis of bacterial infections [30,31]. Several studies have reported that this parameter improves the diagnosis of sepsis in critically-ill patients [32-36] and is useful in monitoring antibiotic therapy in infected patients in an Intensive Care Unit (ICU) [37,38]. However, procalcitonin appears not to have good diagnostic accuracy in predicting pneumonia in patients treated in Primary Care [39], while CRP is better than procalcitonin in monitoring antibiotic therapy in patients with pulmonary disease [29]. Several patients were affected with concomitant infectious diseases which would cause an increase in inflammatory markers. As such, the underlying pathology is likely to be a potential source of the high grade inflammation documented by high CRP and procalcitonin concentrations. Most infections are far too

complex to be reduced to a single cutoff point of any biomarker, and the performance of any one of them may vary depending on the type of infection, the severity, and the presence, or not, of underlying diseases. In our study, the extent of infection is relatively low, as compared with reports in patients receiving critical-care in ICU, and the serum concentrations of procalcitonin observed in our study were not very high. In this particular setting, our results show that TBBLase and paraoxonase specific activities have a better discriminatory performance than procalcitonin and CRP.

An evident question is why we chose to measure PON1-related parameters, instead of PON2 or PON3, when there is more evidence in the literature for the involvement of these other two molecules in the protection against infection [24]. Essentially, our reasons were practical: PON2 is an exclusively intracellular enzyme that cannot be measured in the circulation. PON3, on the other hand, is found in blood bound to high-density lipoprotein particles and the method for its measurement involves hydrolysis of simvastatin to β,δ-dihydroxyacid simvastatin which is, then, measured spectrophotometrically following high-performance liquid chromatography separation [40]. These methods are cumbersome and are rarely available in a routine clinical laboratory. Conversely, serum TBBLase or paraoxonase activities may be analyzed in standard microplates in a few minutes, the analyses are relatively cheap and do not require any specific equipment (with the exception of a fume hood to manipulate the paraoxon substrate currently used in measuring paraoxonase activity). An additional advantage of TBBLase is that it is not strongly influenced by PON1 gene polymorphisms, as is paraxonase [17]. Serum PON1 concentration may also be analyzed easily using ELISA. Although we chose to employ our in-house method, several commercial companies have launched reliable kits for the measurement of PON1 concentration.

5. Conclusion

Our study needs to be considered preliminary. The population studied is heterogeneous, and the participants differ with respect to etiology and comorbidities. Additionally, the number of participants is relatively low. Despite these caveats, our results suggest that the measurement of serum TBBLase, or paraoxonase, specific activities may contribute to the diagnosis of ACI in patients carrying a CVC. We highlight the need for studies conducted with a greater number of patients to confirm this hypothesis.

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Conflict of interest statement

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Authors'contributions

SI, AC, JJ and JC designed the study. SI and AC conducted the clinical aspects, while AGH, IP, FB, IGF, JMS and JJ conducted the laboratory aspects of the study. Data management was by SI and AGH. JC wrote the first draft of the manuscript and all authors contributed to subsequent drafts. All authors read and approved the final manuscript.

Supplementary material

Table S1. Results of selected biochemical variables in patients with a CVC, and classified according to the severity of the catheter-related infection (CRI).

Table S2. Results of PON1-related variables in patients with a CVC, segregated according to whether or not they received antibiotic treatment.

Table S3. Results of PON1-related variables in patients with an ACI, segregated according to whether or not they received antibiotic treatment.

Table S4. Genotype frequencies of the $PON1_{192}$ and $PON1_{55}$ gene polymorphisms in the control group and in patients with an indwelling central venous catheter.

Table S5. Paraoxonase and TBBLase activities in the patients and in the control group segregated according to the $PON1_{192}$ gene polymorphism isoforms.

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Figure legends

Figure 1 Comparisons of PON1-related variables *versus* procalcitonin and C-reactive protein (CRP) concentrations in patients with an indwelling central venous catheter. The r values are the exponential correlation coefficients, except for the correlation between TBBLase activity and CRP which is linear.

Figure 2 Results of selected biochemical variables in the control group and in patients with or without catheter-related infection (CRI)

Figure 3 Results of selected biochemical variables in the control group and in patients with or without an acute concomitant infection (ACI)

Figure 4 Receiver operating characteristics plots for selected biochemical variables in patients segregated with respect to acute concomitant infection (left panel) or catheter infection (right panel)