Serum calprotectin levels in elderly males and females without bacterial or viral infections

Tom Nilsen, Johan Sundström, Lars Lind, Anders Larsson

Abstract

Objectives: Calprotectin is released from activated leukocytes and calprotectin can thus be used as a marker for leukocyte activation. Faeces calprotectin is not only used as a marker for inflammatory bowel disease but can also be used to detect leukocyte activation in other body fluids. The aim of the present study was to study serum calprotectin levels in non-infected elderly individuals to establish reference intervals for the marker.

Methods: Serum calprotectin was analyzed by immunoturbidimetry in 75 year old females and males without known infections. Individuals with CRP > 20 mg/L were excluded as this could indicate a subclinical infection. The calprotectin levels in the remaining 713 individuals were used to calculate reference values for this population. The Spearman rank correlations between calprotectin and 27 other laboratory biomarkers were also investigated.

Results: There was a strong positive Spearman rank correlation between calprotectin and CRP (p < 0.000001) and alkaline phosphatase (p < 0.000001). There were also significant negative correlations between calprotectin and ApoA1 and direct HDL-cholesterol.

Conclusions: The reference interval for serum-calprotectin for all study subjects was 0.3–2.6 mg/L. Leukocyte alkaline phosphatase contributes to serum alkaline phosphatase levels.

Introduction

Calprotectin is a calcium and zinc binding protein found predominantly in the cytosol of neutrophils where it accounts for 30–40% of the protein content [1,2]. Calprotectin is a complex consisting of one S-100:A8 and one S-100:A9 molecule or multimers of these molecules [3]. The protein is released when the neutrophils are activated and the granule content released [4]. Calprotectin has several functions both intracellularly and extracellularly. The intracellular functions involve the activation of the neutrophilic NADPH-oxidase and modulation of the cell cytoskeleton during migration of phagocytes [5–7]. The extracellular effects include pro-inflammatory, antimicrobial, oxidant-scavenging and apoptosis-inducing activities [8,9]. In vitro studies show that calprotectin has both bacteriostatic and fungistatic properties and is resistant to enzymatic degradation. Calprotectin stimulates recruitment of leukocytes, promotes cytokine and chemokine production, and regulates leukocyte adhesion and migration. Over the past years, faecal calprotectin has been shown to be a good biological marker of intestinal inflammation, especially in inflammatory bowel disease (IBD) [10,11]. When neutrophils and monocytes are stimulated they respond by secreting calprotectin into the extracellular fluid [12]. Calprotectin is a potent acute phase reactant with increases of more than 100 fold during inflamed conditions [13]. Even if calprotectin has mainly been used as a marker for IBD, it has been proposed as a biomarker for different inflammatory diseases due its potential role in the pathophysiology of inflammation.

Elderly patients seek health care more often than those who are 20–50 years of age. They are more susceptible to infections and these infections are a common cause of increased morbidity and mortality. The infections often have altered clinical presentations, which may make it difficult to diagnose the infections early. Thus, biomarkers for infectious diseases are especially important in this patient group. It is thus important to establish reference intervals in elderly populations for biomarkers that could be used to diagnose infectious diseases. Increased levels of serum calprotectin have been reported in a number of inflammatory related disorders including patients with rheumatoid arthritis, systemic lupus erythematosus (SLE), vasculitis, glomerulonephritis, sepsis and other bacterial infections [14].

Calprotectin is often measured by ELISA which usually is associated with rather long test turn-around time especially if the method is microtitre plate based and the laboratory tries to fill the whole plate with samples to minimize reagent costs. The development of a particle enhanced turbidimetric assay that can be used on chemistry analyzers allows much shorter test turn-around time.
The aim of the study was to define reference values for serum calprotectin in an elderly population. We also wanted to study the correlation between serum calprotectin and some other frequently used laboratory markers to investigate which of these markers could influence the serum calprotectin levels. The correlation between the calprotectin results and serum alanine aminotransferase, albumin, alkaline phosphatase, apolipoprotein A1, apolipoprotein B, bilirubin, calcium, chloride, cholesterol, creatinine, creatine kinase, C-reactive protein, direct HDL-cholesterol, γ-glutamyltransferase, iron, lactate dehydrogenase (LDH), pancreatic lipase, magnesium, phosphate, potassium, sodium, transferrin, triglycerides, uric acid, and urea was thus also investigated.

Materials and methods

Subjects

Eligible for the study at the initial inclusion was all subjects aged 70 years living in Uppsala, Sweden. The subjects were chosen from the register of community living and were invited in a randomized order from the start of the study in April 2001 to the last-included subject in June 2005. The subjects received an invitation letter within 1 month of their 70th birthday for the standardization of age. Of the 2025 subjects invited, 1016 subjects were investigated giving a participation rate of 50.1%. The study is part of the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study [15]. The study was performed in accordance with the ethical principles for medical research of the Declaration of Helsinki.

These subjects were reinvestigated at age 75. At the reinvestigation, 821 participated and provided blood samples but for the study we only had serum samples from 731 individuals. Blood samples were collected from 2006 to 2009 in vacutainer tubes without additives. The samples were centrifuged, and the sera were frozen at −80 °C in multiple tubes. The calprotectin assays were performed in 2013 and the other chemistry markers were analyzed in 2010. Separate tubes were used for the measurements in 2010 and 2013. Calprotectin retains the antigenic even in faeces after several days of storage at room temperature indicating that it is a stable molecule. There are no long time data on storage of calprotectin at −80 °C but calprotectin is stable for at least 6 months at −20 °C without any loss of antigen activity [16,17]. The marker should thus also be stable at −80 °C in the present study. The Ethics Committee of the University of Uppsala approved the study, and the participants gave informed consent prior to blood sampling.

Baseline investigation

The participants were asked to answer a questionnaire about their medical history, smoking habits, and regular medication. All subjects were investigated in the morning after an overnight fast. No medication or smoking was allowed after midnight. During the investigation, the subjects were supine in a quiet room maintained at a constant temperature.

Calprotectin assay

Serum calprotectin was analyzed on a BS380 instrument (Mindray, Shenzhen, China) with calprotectin reagents from Gentian (Moss, Norway). The measurements were performed using the following instrument settings: wavelength 505 nm, 150 μL reagent 1, 2.5 μL sample, 50 μL reagent 2 and a spline calibration method. The total CV for the calprotectin method was: 2.5% at 1.2 mg/L. The calprotectin assay is a particle-enhanced turbidimetric immunoassay (PETIA) utilising affinity purified avian antibodies bound to particles. The antibody coated particles bind to calprotectin present in the samples leading to an aggregation of the particles and an increased turbidity. The increase in turbidity is proportional to the amount of calprotectin in the samples.

Other clinical chemistry tests

Serum alanine aminotransferase (ALT) (EC 2.6.1.2, reagent: 1655281), albumin (reagent: 8196057), alkaline phosphatase (EC 3.1.3.1, reagent: 1053180), apolipoprotein A1 (reagent: 6801737), apolipoprotein B (reagent: 6801738), bilirubin (reagent: 8383051), calcium (reagent: 1450261), chloride (reagent: 8445207), cholesterol (reagent: 1669829), creatinine (reagent: 8141947), creatine kinase (reagent: 8479396), C-reactive protein (reagent: CRP Vario, Abbott Laboratories, Abbott Park, IL, USA), direct HDL-cholesterol (reagent: 6801895), γ-glutamyltransferase (GGT) (EC 2.3.2.2, reagent: 8257289), iron (reagent: 1515808), lactate dehydrogenase (LDH) (reagent: 838489), pancreatic lipase (EC 3.1.1.3, reagent: 1668409), magnesium (reagent: 825593), phosphate (reagent: 1513209), potassium (reagent: 8157596), sodium (reagent: 8379034), transferrin (reagent: 6801767), triglycerides (reagent: 1336544), uric acid (reagent: 1943927), and urea (reagent: 8102204) measurements were performed on an Ortho Vitros FS 5.1 (Ortho Clinical Diagnostics, Rochester, NY, USA) and reported using SI units. If not stated otherwise within the brackets, the reagents were all obtained from Ortho Clinical Diagnostics. The assays were performed at the Department of Clinical Chemistry, Kalmar, Sweden. Swedac (Borås, Sweden) accredits the laboratory according to ISO 17025 [18].

Statistical calculations

Calculations of reference intervals were performed by bootstrap estimation utilising RefVal. 4.0 (Department of Clinical Chemistry, Rikshospitalet, N-0027 Oslo, Norway). RefVal fulfils the recommendations of the International Federation of Clinical Chemistry on the statistical treatment of reference values [19,20]. Spearman rank correlation was used to investigate associations between calprotectin and other studied biomarkers and comparisons between groups were performed with Mann–Whitney U test (Statistica, StatSoft, Tulsa, OK, USA).

A significance level of p < 0.001 was used for Spearman rank correlations to compensate for multiple testing of correlations (approximately 30 associations were studied).

Results

Reference interval for calprotectin

All study persons with CRP values ≤20 mg/L were included in the reference intervals (n = 713). No significant differences in calprotectin levels were seen with the Wilcoxon U test between patients with and without cardiac failure, diabetes, stroke (at 70 or 75 years of age) or MI (at 70 years of age). There was a weak positive Spearman rank correlation between calprotectin and body mass index (BMI) (R_s = 0.09; p = 0.02).

Reference intervals were calculated separately for females and males and combined.

The reference interval for calprotectin for male subjects (n = 347) was 0.3 (0.1–0.4) to 2.6 (2.1–3.0) mg/L while the corresponding reference interval for female subjects (n = 366) was 0.3 (0.2–0.4) to 2.6 (2.3–3.0) mg/L. There was no significant difference between sexes (p = 0.61, Wilcoxon U test). We thus also calculated a reference interval for calprotectin for all study subjects (n = 713): 0.3 (0.2–0.3) to 2.6 (2.3–2.8) mg/L (Fig. 1).

Correlations between calprotectin and other clinical chemistry markers

There were significant (p < 0.001) Spearman rank correlations between calprotectin and alkaline phosphatase (R_s = 0.19; p < 0.000001) (Fig. 2), CRP (R_s = 0.29; p < 0.000001), direct HDL cholesterol.
(RS = −0.14; p = 0.00018) and apolipoprotein A1 (RS = −0.13; p < 0.00089) (Table 1). Other correlations below p = 0.05, but not significant according to our defined significance level, were observed between calprotectin and iron (RS = −0.12; p = 0.0011), lactate dehydrogenase (RS = 0.12; p = 0.0016), triglycerides (RS = 0.12; p = 0.0016), and apolipoprotein B/apolipoprotein A1 ratio (RS = 0.11; p = 0.0036). There were no significant correlations between calprotectin and alanine aminotransferase, bilirubin or γ-glutamyltransferase. There was a weak positive correlation between calprotectin and body mass index (BMI) (RS = 0.11; p = 0.0036).

**Discussion**

To be able to interpret test results correctly it is important to have appropriate reference intervals. Without such values there is a risk that the test results are misinterpreted.

The reference intervals should also be representative for the patient groups for whom they are intended for. Most patients seeking health care are elderly but often the reference intervals lack individuals older than 60–65 years of age. We thus decided to calculate reference values for serum calprotectin in an elderly population consisting of 75 year old individuals. Individuals with CRP values >20 mg/L were excluded from the reference interval calculations as the increased CRP values could indicate subclinical infections that could result in increased calprotectin values.

Even after exclusion of individuals with CRP values >20 mg/L there was a significant association between calprotectin and CRP. This was anticipated as both markers increase in response to pro-inflammatory cytokines [21,22].

There was also a significant correlation between calprotectin and alkaline phosphatase, a marker usually used for the diagnosis of liver disorders and particularly bile ducts obstruction. Other organs often attributed with high alkaline phosphatase levels are kidney, bone, and the placenta. The leukocytes are rarely mentioned in textbooks

<table>
<thead>
<tr>
<th>Analyte</th>
<th>R</th>
<th>p-Value</th>
<th>mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine aminotransferase (μkat/L)</td>
<td>0.06</td>
<td>0.09</td>
<td>0.38 ± 0.14</td>
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<tr>
<td>Alkaline phosphatase (μkat/L)</td>
<td>−0.05</td>
<td>0.19</td>
<td>44.3 ± 3.63</td>
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<td>Apolipoprotein A1 (g/L)</td>
<td>−0.13</td>
<td>0.00089</td>
<td>1.69 ± 0.33</td>
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<td>Apolipoprotein B (g/L)</td>
<td>0.03</td>
<td>0.36</td>
<td>1.08 ± 0.25</td>
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<tr>
<td>Apolipoprotein B/apolipoprotein A1</td>
<td>0.11</td>
<td>0.0036</td>
<td>0.66 ± 0.19</td>
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<tr>
<td>Bilirubin (μmol/L)</td>
<td>−0.04</td>
<td>0.24</td>
<td>5.98 ± 3.79</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>−0.08</td>
<td>0.02</td>
<td>2.39 ± 0.75</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>0.03</td>
<td>0.42</td>
<td>106 ± 2.74</td>
</tr>
<tr>
<td>Cholesteryl (mmol/L)</td>
<td>−0.04</td>
<td>0.24</td>
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<td>HDL-cholesterol (mmol/L)</td>
<td>−0.14</td>
<td>0.0018</td>
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<td>LDL-cholesterol (mmol/L)</td>
<td>−0.01</td>
<td>0.84</td>
<td>3.23 ± 0.97</td>
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<tr>
<td>Creatinine (μmol/L)</td>
<td>0.04</td>
<td>0.27</td>
<td>81.0 ± 27.9</td>
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<td>Creatine kinase (μkat/L)</td>
<td>−0.07</td>
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<td>1.58 ± 1.05</td>
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<td>C-reactive protein (CRP) (mg/L)</td>
<td>0.29</td>
<td>&lt;0.00001</td>
<td>3.88 ± 5.97</td>
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<td>γ-Glutamyltranspeptidase (GGT) (μkat/L)</td>
<td>0.07</td>
<td>0.065</td>
<td>0.64 ± 0.79</td>
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<td>Iron (μmol/L)</td>
<td>−0.12</td>
<td>0.0011</td>
<td>18.8 ± 5.65</td>
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<td>Lactate dehydrogenase (LDH) (μkat/L)</td>
<td>0.12</td>
<td>0.0016</td>
<td>2.34 ± 0.45</td>
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<td>Lipase (μkat/L)</td>
<td>−0.02</td>
<td>0.57</td>
<td>2.25 ± 1.35</td>
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<td>Magnesium (mmol/L)</td>
<td>−0.05</td>
<td>0.21</td>
<td>0.82 ± 0.07</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>−0.05</td>
<td>0.18</td>
<td>1.17 ± 0.14</td>
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<td>Potassium (mmol/L)</td>
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<td>Transferrin (g/L)</td>
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<td>Triglycerides (mmol/L)</td>
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<td>0.0016</td>
<td>1.43 ± 0.67</td>
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<tr>
<td>Urate (μmol/L)</td>
<td>0.07</td>
<td>0.05</td>
<td>320 ± 74.7</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>0.07</td>
<td>0.06</td>
<td>650 ± 1.87</td>
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<tr>
<td>White blood cell count (109/L)</td>
<td>0.01</td>
<td>0.82</td>
<td>5.84 ± 1.68</td>
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</table>
as a source for alkaline phosphatase. Leukocyte alkaline phosphatase (LAP) or neutrophil alkaline phosphatase is found in white blood cells and LAP staining of leukocytes is used as a diagnostic tool [23,24]. LAP is an enzyme present in normal neutrophils, but present at very low concentrations in malignant neutrophils [25]. Increased LAP levels are seen in the leukemoid reaction [26]. This term describes an elevated white blood cell count that is a physiological response to stress or infection. Thus the association between calprotectin and LAP observed in this study is most likely due to leukocyte alkaline phosphatase (LAP) released from neutrophils when activated. The absence of significant correlations between calprotectin and any of the liver markers, alanine aminotransferase, bilirubin and γ-glutamyltransferase, indicates that the ALP increase is not due to liver damage.

We also observed significant associations between calprotectin and direct HDL-cholesterol and apolipoprotein A1. Apolipoprotein A1 is part of the HDL particle and both HDL-cholesterol and apolipoprotein A1 are both used as markers for the HDL particle. Apolipoprotein A1 has well described anti-inflammatory effects and can also influence the recruitment of neutrophils [27]. Mice lacking apolipoprotein A1 displayed enhanced recruitment of neutrophils to the air-space while treatment with apolipoprotein A1 mimetic peptides reduced airway neutrophilia [28]. These findings are in agreement with our negative correlation between calprotectin and HDL-cholesterol or apolipoprotein A1. The study is based on data from elderly males and females living in Sweden. The problems of generalising these results to other age-groups and ethnic groups have to be acknowledged especially the generalisation of the results to areas with higher rates of infections than Sweden. In conclusion: We report reference intervals for serum calprotectin in elderly males and females. There were no significant differences between males and females and we thus recommend a reference interval based on both males and females (0.3 to 2.6 mg/L). There was a significant association between calprotectin and alkaline phosphate indicating that the release of alkaline phosphatase from neutrophils may contribute to the alkaline phosphate levels found in patient samples. It is important to be aware of this and to not assume that a slightly elevated alkaline phosphate level is always due to bile duct obstruction. The clinical usefulness of measuring serum calprotectin in specific patient groups remains to be evaluated.

Declaration of interest

Tom Nilsen is employed by Gentian, Moss, Norway.

Acknowledgments

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\[1\] Yui S, Nakatani Y, Mikami M. Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. Biol Pharm Bull 2003;26:753–60.


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