Evaluation of neopterin as a prognostic factor in patients with beta-thalassemia, in comparison with cytokines and immunoglobulins

OBJECTIVE Investigation of whether the serum level of neopterin can be used as a marker in patients with β-thalassemia, evaluation of its clinical significance and correlation with other laboratory and clinical parameters, including the cytokines interleukin-4 (IL-4), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), high sensitive C-reactive protein (hs-CRP) and immunoglobulins.

METHOD Study was made of 20 healthy subjects and 56 patients with β-thalassemia, ranging in age from 3 to 25 years. The serum levels of human neopterin, IL-4, IL-6 and TNF-α were determined by the ELISA method, and of hs-CRP and immunoglobulins IgA, IgM, IgG and IgG subclasses IgG1, IgG2, IgG3 and IgG4 by the nephelometric technique.

RESULTS The serum levels of neopterin, IL-6, TNF-α and hs-CRP were significantly higher in patients with β-thalassemia than in normal subjects, but the level of IL-4 was the same in the two groups. The levels of immunoglobulins IgA and IgG, as well as IgG subclasses IgG1, IgG2, IgG3 and IgG4 were significantly higher in patients with β-thalassemia, but the level of IgM was the same as in normal subjects.

CONCLUSIONS The increased serum level of neopterin found in patients with β-thalassemia may be due to inflammation occurring with frequent blood transfusions. The increase in neopterin was associated with elevation in hs-CRP, cytokines IL-6 and TNF-α, and immunoglobulins IgA, IgG and IgG subclasses, and was not affected by sex or age.

Key words
β-thalassemia
Cytokines
hs-CRP
Immunoglobulin
Neopterin

A wide spectrum of immune abnormalities has been described in reports of numerous studies on patients with β-thalassemia who have received multiple blood transfusions. The abnormalities observed to date are both quantitative and functional, and involve several components of the immune response.

It has been suggested that a variety of factors, including splenectomy, iron overload, repeated exposure to foreign antigens at the time of blood transfusion and the use of chelating agents, may induce profound deleterious effects on the immune system.

An increase in the serum level of neopterin has been associated with diseases involving the cellular immune response (e.g., viral infections and infections by intracellular
bacteria or parasites, autoimmune diseases and certain malignancies).  

Inflammatory biomarkers, including C-reactive protein (CRP) and interleukin-6 (IL-6) are increased in various inflammatory conditions and have been found useful in studying thalassemia and other disease states, including heart disease.  

The aim of this study was to examine whether the serum level of neopterin can be used as a marker in patients with β-thalassemia major, and to evaluate its clinical significance and its correlation with other laboratory and clinical parameters, specifically the cytokines interleukin-4 (IL-4), IL-6 and tumor necrosis factor-alpha (TNF-α), high sensitive CRP (hs-CRP) and immunoglobulins (IgA, IgM, IgG and the IgG subclasses IgG1, IgG2, IgG3 and IgG4).

**MATERIAL AND METHOD**

This study was conducted on 76 subjects, ranging in age from 3 to 25 years, after securing the informed written consent of themselves or their guardians, according to rules of the medical research ethics committee at the national research centre (number: 10–172). The subjects were divided into two groups: Group I consisted of 20 healthy subjects matched in age and gender with the subjects in Group II who were 56 patients with β-thalassemia major.

**Clinical evaluation**

A detailed registration sheet was designed to include personal history, pedigree construction, as well as clinical and genetic history, with special emphasis on a family history of hereditary blood disorder (HBD), and details of the clinical examination (some congenital anomalies with seemingly idiopathic cytopenia, even when mild, warrant consideration of a possible inherited bone marrow failure syndrome (IBMFS)).

The results of all investigations and follow up were incorporated on the sheet, including anthropometric measurements; height, head circumference and weight, with pubertal assessment.

**Collection of blood samples and processing**

A sample of 5 mL of venous blood was collected by a well-trained nurse from each subject. In the case of patients with β-thalassemia, the sample was taken just before a scheduled transfusion of packed red blood cells.

Vacutainer plain tubes were left for short time to allow the blood to clot, after which clear serum samples were obtained by centrifugation (B. Bran-Sigma 2K15, USA) at 4,000 rpm for 10 minutes. The separated serum was sealed and stored at -20 ºC until the time of analysis. The frozen serum samples were thawed at 4–8 ºC, then mixed by gentle shaking at room temperature prior to determination of the levels of IL-4, IL-6, TNF-α, neopterin, hs-CRP and immunoglobulins (IgG, IgA, IgM and IgG subclasses IgG1, IgG2, IgG3 and IgG4).

**Estimation of human serum neopterin**

Human neopterin was determined quantitatively by ELISA in serum using the kit of IBL International GmbH, Flughafenstrasse 52A, D-22335 Hamburg, Germany using EL×800 Absorbance Microplate Reader (Bio-TEC, USA) (96-A microplate reader RES9321, Dr Spangenberg 2012), according to the manufacturer instructions.

**Measurement of serum cytokines**

The cytokines IL-4, IL-6 and TNF were measured by AviBion Human ELISA kits, Oorgenium Laboratories Business Unit, Vantaa, Finland, according to the manufacturer’s recommendations.

**Measurement of serum high sensitive C-reactive protein and immunoglobulins**

The measurement of human hs-CRP and immunoglobulins (IgA, IgM, IgG and the IgG subclasses IgG1, IgG2, IgG3 and IgG4) in serum was performed using the nephelometric technique. Product Code: ZK044.LR, Minineph TM, The Binding Site Ltd, PO Box 11712, Birmingham, B14 4ZB, UK.

**Statistical methods**

Data were expressed as median with range (min–max). Statistical significance of differences was analyzed using the Statistical Package for Social Science (SPSS), version 15.0, using the non-parametric Mann-Whitney test for comparison of median values. P values of <0.05 were considered statistically significant. The correlation coefficient (r), which is a measure of the degree of closeness of the linear relationship between two variables (X and Y), was determined; r always lies between -0.01 and +0.01.

**RESULTS**

Table 1 shows comparison of the levels of the serum parameters in the groups of patients with β-thalassemia and healthy subjects. The median serum levels of IL-6 (51.5, range 25–120 pg/mL) and TNF-α (49.50, range 28–100 pg/mL) in the patients with β-thalassemia were significantly higher than those of the healthy subjects (40.50, range 21–72 pg/mL, and 37.50, range 16–70 pg/mL, respectively). There was no difference in the serum IL-4 levels between the patients with β-thalassemia and the healthy subjects. The median serum levels of IgA and IgG of the patients with β-thalassemia were significantly higher than those of the healthy subjects (2.3, range 0.7–5.7 g/L vs 1.7, range 0.9–2.4 g/L, respectively and 14.5, range 6.0–20 g/L vs 10.1,
The serum IgM levels were the same in the two groups. In addition, the median serum levels of all the IgG subclasses were significantly higher in the patients with β-thalassemia (IgG1: 9,300, range 2,614–10,900 vs 6,400, range 5,100–7,900 mg/L; IgG2: 5,450, range 2,500–6,600 vs 4,750, range 3,000–5,500 mg/L; IgG3: 879, range 143–1,950 vs 470, range 146–1,320 mg/L; IgG4: 249.5, range 58–815 vs 137, range 58–380 mg/L).

The median serum level of neopterin was statistically significantly higher in the patients with β-thalassemia major (19.0 nmoL/L, range 10.0–33.0 nmoL/L) than in the healthy subjects (9.0 nmoL/L, range 6.0–12.8 nmoL/L).

The median serum level of hs-CRP was also significantly higher in patients with β-thalassemia major (4.2 mg/L, range 3.6–4.4 mg/L) than in the healthy subjects (3.6 mg/L, range 3.6–4.4 mg/L).

As shown in table 2, a statistically significant correlation was demonstrated between serum levels of neopterin and hs-CRP in the patients with β-thalassemia (r=0.4, p<0.05).

### DISCUSSION

Patients with β-thalassemia major present many problems in addition to their severe anemia, including increased susceptibility to bacterial infections. Infectious complications constitute the second most common cause of mortality and a major cause of morbidity in β-thalassemia, after heart failure.11,12

Several studies on immune competence in β-thalassemia have revealed numerous quantitative and functional defects, involving T and B lymphocytes, immunoglobulin production, neutrophils and macrophages, chemotaxis, and phagocytosis, and changes in the pattern of cytokine production and in the complement system.13,14

### Table 1. Comparison of serum parameters between patients with β-thalassemia and healthy subjects.

<table>
<thead>
<tr>
<th>Parameters (units)</th>
<th>Healthy subjects (n=20)</th>
<th>Patients with β-thalassemia (n=56)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Range (min–max)</td>
<td>Median</td>
</tr>
<tr>
<td>Neopterin (mmol/L)</td>
<td>9.0</td>
<td>6.0–12.8</td>
<td>19.0</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>25.5</td>
<td>8.0–59.0</td>
<td>18.0</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>40.5</td>
<td>25.0–72.0</td>
<td>51.5</td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>37.5</td>
<td>16.0–70.0</td>
<td>49.5</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>3.6</td>
<td>3.6–4.4</td>
<td>4.2</td>
</tr>
<tr>
<td>IgA (g/L)</td>
<td>1.7</td>
<td>0.9–2.4</td>
<td>2.3</td>
</tr>
<tr>
<td>IgM (g/L)</td>
<td>1.1</td>
<td>0.6–1.8</td>
<td>1.2</td>
</tr>
<tr>
<td>IgG (mg/L)</td>
<td>10.1</td>
<td>7.2–12.0</td>
<td>14.5</td>
</tr>
<tr>
<td>IgG1 (mg/L)</td>
<td>6,400</td>
<td>5,100–7,900</td>
<td>9,300</td>
</tr>
<tr>
<td>IgG2 (mg/L)</td>
<td>4,750</td>
<td>3,000–5,500</td>
<td>5,450</td>
</tr>
<tr>
<td>IgG3 (mg/L)</td>
<td>470</td>
<td>146–1,320</td>
<td>879</td>
</tr>
<tr>
<td>IgG4 (mg/L)</td>
<td>137</td>
<td>58–380</td>
<td>249.5</td>
</tr>
</tbody>
</table>

*Statistically significant (p<0.05)

IL-4: Interleukin-4, IL-6: Interleukin-6, TNF-α: Tumor necrosis factor-alpha, hs-CRP: High sensitive C-reactive protein

### Table 2. Correlation between serum levels of neopterin and other parameters in patients with β-thalassemia (n=56).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>R (Pearson correlation)</th>
<th>Significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neopterin ~ IL-4</td>
<td>0.117</td>
<td>0.391 P*</td>
</tr>
<tr>
<td>Neopterin ~ IL-6</td>
<td>0.216</td>
<td>0.109 P*</td>
</tr>
<tr>
<td>Neopterin ~ TNF-α</td>
<td>0.214</td>
<td>0.114 P*</td>
</tr>
<tr>
<td>Neopterin ~ hs-CRP</td>
<td>0.400</td>
<td><em>0.002 P</em></td>
</tr>
<tr>
<td>Neopterin ~ IgA</td>
<td>-0.031</td>
<td>0.819 N†</td>
</tr>
<tr>
<td>Neopterin ~ IgM</td>
<td>-0.061</td>
<td>0.656 N†</td>
</tr>
<tr>
<td>Neopterin ~ IgG</td>
<td>-0.106</td>
<td>0.436 N†</td>
</tr>
<tr>
<td>Neopterin ~ IgG1</td>
<td>-0.012</td>
<td>0.933 N†</td>
</tr>
<tr>
<td>Neopterin ~ IgG2</td>
<td>0.034</td>
<td>0.806 P*</td>
</tr>
<tr>
<td>Neopterin ~ IgG3</td>
<td>-0.158</td>
<td>0.245 N†</td>
</tr>
<tr>
<td>Neopterin ~ IgG4</td>
<td>0.055</td>
<td>0.690 P*</td>
</tr>
</tbody>
</table>

P: Positive correlation, N: Negative correlation, *Correlation significant at the 0.05 level
IL-4: Interleukin-4, IL-6: Interleukin-6, TNF-α: Tumor necrosis factor-alpha, hs-CRP: High sensitive C-reactive protein, Ig: Immunoglobulin
Iron overload has been suggested by some investigators as an important contributing factor in altering the immune parameters in thalassemia.\(^1\)

In the present study, the serum levels of immunoglobulins IgA and IgG, and the IgG subclasses IgG1, IgG2, IgG3 and IgG4 were all statistically significantly higher in patients with β-thalassemia than in healthy subjects but IgM showed insignificant difference. Iron overload results in increased migration of T helper cells to the gut and lymph nodes and this causes an increase in serum immunoglobulin levels in patients with thalassemia.\(^2\) Multiple blood transfusions and continuous immune stimulation could be responsible for a double-faced immune response.\(^1\)

This finding is consistent with the demonstration by Amin and colleagues\(^1\) of an increase in serum levels of IgG and IgA, with no change in serum IgM level in splenectomized patients with β-thalassemia intermedia.

The present study also found that serum levels of IL-6 and TNF-α, but not IL-4, were statistically significantly raised in patients with β-thalassemia compared with healthy subjects. Taking into consideration that IL-4 is predominantly secreted by Th2 cells, a significant impairment of both Th1 and Th2 cytokines might be present in thalasemic patients.

Aggeli and colleagues\(^1\) indicated that an increase in the circulating level of IL-6 among patients with thalassemia is an important component of the pro-inflammatory response, together with IL-8. In the same context, Shfik and colleagues\(^2\) demonstrated increased serum levels of IL-8 and TNF-α in multiply transfused patients with β-thalassemia major.

The increase in IL-6 explains that impairment of the neutrophil and macrophage phagocytic and killing function and the production of certain cytokines are important features. It is known that IL-6 is an important component of the pro-inflammatory response and its high serum level may be relevant in the pathophysiology of β-thalassemia; increased production of IL-6 is probably due to overstimulation of macrophages and might contribute to abnormalities in iron metabolism.\(^1\)

The present study of the serum levels of neopterin in patients with β-thalassemia aimed to evaluate their clinical significance as a diagnostic tool and their correlation with other parameters. In the patients with β-thalassemia, the serum levels of both neopterin and hs-CRP were statistically significantly higher than in the healthy subjects.

A possible explanation for the increased neopterin may be inflammation due to infections or to free radicals formed in response to continuous blood transfusion in these patients.

Neopterin, a pteridine derivative produced by activated macrophages in response to stimulation by interferon-γ, is a marker of both immune activation and coronary artery disease (CAD) activity.\(^3\)

Neopterin enhances inflammatory processes and, together with the pro-inflammatory cytokine TNF-α, stimulates gene transcription for inducible nitric oxide synthesis (iNOS), which results in the production of cytotoxic NO-free radicals.\(^4\) An increased number of activated T cells and higher levels of serum neopterin have been observed in thalassemia, suggesting chronic stimulation of the immune system.\(^1\)

Similar findings have been reported by Berdowska and Zwirska-Korczala\(^5\) who demonstrated that the serum levels of neopterin concentration were higher in patients with β-thalassemia than in a healthy control group.

The cellular immune system could be activated and immunological processes triggered by endotoxins produced by Gram (-) bacteria. Consequently, the activation of T-lymphocytes and formation of INF-α could result in an increase of the concentration of neopterin in body fluids.\(^2\)

Repeated blood transfusion in patients with thalassemia results in the circulation of free iron in the form of non-transferring binding iron (NTBI). NTBI starts to be deposited in the organs, but it is unstable and serves as a precursor for various chemical reactions that produce free reactive radicals. This iron load insult to the tissues has been monitored using hs-CRP as biomarker of inflammation.\(^6\) Studies in vitro have shown that neopterin can enhance the oxidative potential of reactive oxygen species produced from immune competent cells.\(^7\)

In the present study, serum neopterin exhibited a significant correlation with hs-CRP (r=0.40, p=0.002) in patients with β-thalassemia, but not with the other parameters.

This confirms the conclusions of previous studies suggesting that neopterin may provide more comprehensive information regarding risk of cardiovascular events than CRP measurements, particularly when macrophage activation is implicated.\(^2\)

In conclusion, the raised serum levels of neopterin found in patients with β-thalassemia compared with normal healthy control subjects may be due to inflammation which occurs with frequent blood transfusion and is associated with elevation in hs-CRP, the cytokines IL-6 and TNF-α and the immunoglobulins IgA, IgG and subclasses of IgG. This elevation of neopterin was not affected by sex or age.
Αξιολόγηση της νεοπτερίνης ως προγνωστικού παράγοντα σε σχέση με τις κυτταροκίνες και τις ανοσοσφαιρίνες σε ασθενείς με β-μεσογειακή αναιμία

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ΣΚΟΠΟΣ Διεξήχθη μελέτη για τη διερεύνηση του κατά πόσο τα επίπεδα της νεοπτερίνης στον ορό μπορούν να χρησιμοποιηθούν για τον καθορισμό διαφορών μεταξύ φυσιολογικών ατόμων και ασθενών με β-μεσογειακή αναιμία (β-ΜΑ), καθώς και για αξιολόγηση της κλινικής τους σημασίας, αναφορικά με το αν τα επίπεδα της νεοπτερίνης σχετίζονται με άλλες κλινικές και εργαστηριακές παραμέτρους, όπως οι κυτταροκίνες IL-4, IL-6, TNF-α, η hs-CRP και οι ανοσοσφαιρίνες IgA, IgM, IgG, καθώς και IgG1, IgG2, IgG3 και IgG4.

ΥΛΙΚΟ-ΜΕΘΟΔΟΣ Εξετάστηκαν 76 άτομα (20 υγιείς και 56 με β-ΜΑ), ηλικίας 3–25 ετών. Η ανθρώπινη νεοπτερίνη και οι κυτταροκίνες (IL-4, IL-6 και TNF-α) μετρηθηκαν με τη μέθοδο ELISA, ενώ η hs-CRP και οι ανοσοσφαιρίνες (IgA, IgM, IgG, καθώς και IgG1, IgG2, IgG3 και IgG4) στον ορό με νεφελομετρία.

ΑΠΟΤΕΛΕΣΜΑΤΑ Παρατηρήθηκε στατιστικά σημαντική αύξηση της νεοπτερίνης, της IL-6, του TNF-α και της hs-CRP , αλλά όχι στατιστικά σημαντική αύξηση της IL-4 στους ασθενείς με β-ΜΑ σε σύγκριση με τα φυσιολογικά άτομα. Επί πλέον, παρατηρήθηκε στατιστικά σημαντική αύξηση των ανοσοσφαιρινών IgA και IgG, καθώς και των επιπέδων των IgG1, IgG2, IgG3 και IgG4, αλλά όχι στατιστικά σημαντική αύξηση των IgM στους ασθενείς με β-ΜΑ σε σχέση με τα φυσιολογικά άτομα.

ΣΥΜΠΕΡΑΣΜΑΤΑ Η αυξημένη νεοπτερίνη στους ασθενείς με β-ΜΑ σε σχέση με τα φυσιολογικά άτομα μπορεί να οφείλεται σε παρουσία λοιμώξεων λόγω των συχνών μεταγγίσεων, στοιχείο το οποίο εκφράζεται με την αύξηση της hs-CRP , των κυτταροκινών IL-6 και TNF-α, καθώς και των ανοσοσφαιρινών (IgA, IgG και των υποκατηγοριών της IgG). Η εν λόγω αύξηση δεν επηρεάζεται από την ηλικία και το φύλο.

Λέξεις ευερετηρίου: Ανοσοσφαιρίνες, β-μεσογειακή αναιμία, hs-CRP, Κυτταροκίνες, Νεοπτερίνη

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