Serum Neopterin Levels as a Diagnostic Marker of Hemophagocytic Lymphohistiocytosis Syndrome

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The objective of this study was to retrospectively evaluate the utility of serum neopterin as a diagnostic marker of hemophagocytic lymphohistiocytosis (HLH). The medical records of patients diagnosed with HLH (familial and secondary) between January 2000 and May 2009 were reviewed retrospectively, and clinical and laboratory information related to HLH criteria, in addition to neopterin levels, was recorded. A group of 50 patients with active juvenile dermatomyositis (JDM) (who routinely have neopterin levels assessed) served as controls for the assessment of the accuracy, sensitivity, and specificity of neopterin as a diagnostic test for HLH. The Pearson correlation was used to measure the association between serum neopterin levels and established HLH-related laboratory data. Serum neopterin levels were measured using a competitive enzyme immunoassay. During the time frame of the study, 3 patients with familial HLH and 18 patients with secondary HLH were identified as having had serum neopterin measured (all HLH patients were grouped together). The mean neopterin levels were 84.9 nmol/liter (standard deviation [SD], 83.4 nmol/liter) for patients with HLH and 21.5 nmol/liter (SD, 10.13 nmol/liter) for patients with JDM. A cutoff value of 38.9 nmol/liter was 70% sensitive and 95% specific for HLH. For HLH patients, neopterin levels correlated significantly with ferritin levels ($r = 0.76, P = 0.0007$). In comparison to the level in a control group of JDM patients, elevated serum neopterin was a sensitive and specific marker for HLH. Serum neopterin has value as a diagnostic marker of HLH, and prospective studies are under way to further evaluate its role as a marker for early diagnosis and management of patients.

Hemophagocytic lymphohistiocytosis syndrome (HLH) is a rare clinical condition characterized by prolonged fevers in association with hepatosplenomegaly, cytopenias, coagulopathy, and central nervous system (CNS) manifestations. HLH results from a pathological activation of macrophages leading to hyperproduction of cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (9), that is believed to be the cause of many of the clinical symptoms. HLH is currently classified into a familial form, affecting primarily infants and young children, and a secondary form, which usually occurs in older children. The secondary form of HLH is associated with autoimmune disorders, infections, and malignancies. Macrophage activation syndrome (MAS) is a term that has been used by rheumatologists and refers to the secondary form of HLH seen in the context of rheumatic disorders (13). Familial HLH is an invariably fatal disease curable only with bone marrow transplant. MAS or the secondary form of HLH also has a relatively high mortality rate (8 to 22%) even if treated appropriately (16).

The current diagnostic and therapeutic guidelines were recently reviewed in the Histiocyte Society Treatment and Guideline Protocol (HLH-2004) (8). These diagnostic criteria, summarized in Table 1, do not distinguish between familial or secondary HLH. The diagnosis requires that five of the following eight criteria are met: fever, splenomegaly, cytopenias, hypertriglyceridemia and/or hypofibrinogenemia, hemophagocytosis in bone marrow, spleen, or lymph nodes with no evidence of malignancy, low or absent natural killer (NK) cell activity, hyperferritinemia, and elevated soluble CD25 (i.e., soluble interleukin 2 receptor alpha [sIL-2Rα]) (8). Other findings, such as liver dysfunction with elevated serum transaminases, coagulopathy, and neurological symptoms, are often seen in patients with HLH (2–4) but are not included in the current HLH-2004 diagnostic guidelines (6, 8).

Familial HLH is inherited in an autosomal recessive pattern. Three genes have been found to underlie more than 50% of the familial HLH cases worldwide: PRFI, encoding perforin, a major cytotoxic protein; UNC13D, encoding the MUNC 13-4 protein, which is involved in the exocytosis of perforin-bearing cytotoxic granules; and STX11, encoding the protein t-SNARE syntaxin 11 involved in vesicular transport (6, 12). HLH has been associated with other autosomal recessive immunodeficiencies, including Chediak-Higashi syndrome caused by mu-
The diagnosis of HLH can be established by one or both of the guidelines described in the table, which has been adapted from reference 8 with permission of the publisher.

| Guideline | Description |
|-----------|-------------|
| 1......... | Molecular diagnosis consistent with HLH |
| 2......... | Diagnostic criteria for HLH fulfilled (≥5 of the 8 criteria below) |
| (a) Fever | |
| (b) Splenomegaly | |
| (c) Cytophenias (affecting at least 2 of the following 3 parameters in the peripheral blood) Hemoglobin, <90 g/liter (≤100 g/liter in infants <4 wk old) Neutrophils, <1.0 × 10⁹/liter Fibrinogen, ≤1.5 g/liter |
| (d) Hypertriglyceridemia and/or hypofibrinogenemia Fasting triglycerides, ≥3.0 mmol/liter (≥265 mg/dl) Fibrinogen, ≤1.5 g/liter |
| (e) Hemophagocytosis in bone marrow, spleen, lymph nodes, or cerebrospinal fluid; no evidence of malignancy |
| (f) Low or absent NK cell activity (according to local laboratory reference) |
| (g) Elevated ferritin (≥500 U/liter) |
| (h) Soluble CD25 (i.e., soluble IL-2R) above normal limits for age |

a The diagnosis of HLH can be established by one or both of the guidelines described in the table, which has been adapted from reference 8 with permission of the publisher.
### Table 2: Clinical data for patients with HLH

| Patient | Primary diagnosis | Neopterin level at diagnosis (nmol/liter) | Fever | Splenomegaly | Cytopenias | Increased triglycerides | Decreased fibrinogen | Increased ferritin | Increased D-dimer | Hemophagocytosis | sCD25 | NK cell activity | Gene mutation | Stem cell transplant status |
|---------|-------------------|------------------------------------------|-------|--------------|-----------|-------------------------|------------------|-------------------|-----------------|----------------|--------|----------------|-------------|--------------------------------|
| 1       | AHLH              | 26.4                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Alive (A)                                      |
| 2       | AHLH              | 39.7                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Alive (A)                                      |
| 3       | SJIA              | 87                                       |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 4       | SLE               | 23.2                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Alive (A)                                      |
| 5       | AHLH              | 250                                      |       |              |           |                         |                  |                   |                 |                |        |                |            | Alive (A)                                      |
| 6       | XLP               | 71.5                                     |       |              |           |                         |                  |                   |                 |                |        |                | SH2D1A     | Alive (A)                                      |
| 7       | AHLH              | 72                                       |       |              |           |                         |                  |                   |                 |                |        |                |            | Alive (A)                                      |
| 8       | SJIA              | NA                                       |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 9       | SJIA              | 34.4                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 10      | SLE               | 36                                       |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 11      | SJIA              | 86.2                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 12      | JIA               | 34                                       |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 13      | SJIA              | 48.2                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 14      | MCTD              | 32.2                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 15      | AHLH              | 63.9                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Alive (A)                                      |
| 16      | SJIA              | 347.7                                    |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 17      | Sarcoid           | 94.7                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 18      | SJIA              | 96                                       |       |              |           |                         |                  |                   |                 |                |        |                |            | Not available (NA)                            |
| 19      | KD/FHLH           | 98.5                                     |       |              |           |                         |                  |                   |                 |                |        |                | RAB27A     | Alive (A)                                      |
| 20      | FHLH              | 71.8                                     |       |              |           |                         |                  |                   |                 |                |        |                | UNC13D     | Not available (NA)                            |
| 21      | AHLH              | 63.8                                     |       |              |           |                         |                  |                   |                 |                |        |                |            | Alive (A)                                      |

Characteristics are indicated as present (+) or absent (-). Abbreviations: NA, not available; AHLH, acquired HLH; SJIA, systemic juvenile idiopathic arthritis; SLE, systemic lupus erythematosus; XLP, X-linked lymphoproliferative syndrome; JIA, juvenile idiopathic arthritis; MCTD, mixed connective tissue disease; KD, Kikuchi disease; FHLH, familial HLH.

The gene mutation is identified if present.

A, alive; D, dead.
74% were female. The majority of patients in both groups were white (Table 3), which represents the demographic of our patient population more than an increased susceptibility in any particular ethnicity.

The patients with familial HLH were treated with a combined steroid/immunosuppression protocol, followed by stem cell transplant, with the exception of one child who had UNC13D mutations and who died prior to transplant. Patients with HLH and a rheumatic condition were treated with corticosteroids and cyclosporine and went into remission, except for the patient with sarcoidosis, who died secondary to multiorgan failure. Two of the six patients with no known underlying disorder underwent stem cell transplant due to lack of control of HLH symptoms following immunosuppressant medication.

Serum neopterin levels in the HLH group were significantly higher than those observed in the JDM control group (mean, 83.9 nmol/liter versus 20.8 nmol/liter, respectively) (Fig. 1). The minimum level of serum neopterin in the HLH group was 23.2 nmol/liter, and the maximum level was 347.7 nmol/liter. In comparison, the minimum level in the JDM group was 5.7 nmol/liter, and the maximum level was 43 nmol/liter. In the HLH patients, there were no significant associations with any of the HLH laboratory criteria (data not shown) other than the level of ferritin, which showed a significant correlation with the neopterin level at the time of diagnosis ($P = 0.0007, r = 0.76, n = 16$).

A nonparametric ROC curve using the serum neopterin level at the time of initial diagnosis of HLH was generated. The AUC for serum neopterin levels was 0.93804 (95% confidence interval, 0.86 to 1.00). The ROC analyses indicated that a cutoff value of 38.9 nmol/liter was 70% sensitive and 95% specific for HLH (Fig. 1). At the time of diagnosis (according to the standard criteria [8]), the accuracy of serum neopterin levels ranged from 86% to 100%. The sensitivity and specificity measured at different neopterin cutoff levels ranged from 65% and 100%, respectively, at a cutoff of 48.2 nmol/liter to 100% and 67%, respectively, at the lower cutoff level of 23.2 nmol/liter (summarized in Fig. 2). A prospective study is required to more accurately define a neopterin level appropriate for clinical applications.

## DISCUSSION

HLH is an aggressive and potential fatal syndrome with significant challenges related to both its diagnosis and treatment. It is characterized by an uncontrolled macrophage activation leading to hyperproduction of cytokines. Neopterin is a product of activated human monocyte-derived macrophages and dendritic cells.

Chemically, neopterin is an unconjugated pteridine, which is synthesized from GTP through the GTP cyclohydrolase I pathway. GTP cyclohydrolase I can be induced by IFN-γ in various cells. Neopterin has been reported as a sensitive marker of cell-mediated immune activity (3). Elevated plasma levels of neopterin have been detected in association with cancers (18), during sepsis (3) and numerous infections, including febrile neutropenia (15) and human immunodeficiency virus infection (14), hepatitis B-related chronic liver disease (7), and atherosclerosis (20). Serum neopterin levels have also been reported to correlate with disease activity in JDM patients (4). In addition, Shimizu et al. (17) recently found that serum neopterin concentrations in EBV-related HLH patients were significantly higher than those in patients with MAS/systemic juvenile idiopathic arthritis or Kawasaki disease. The purpose of our study was to evaluate the utility of neopterin as a potential diagnostic marker of HLH. We compared the levels observed in patients with HLH to levels observed in patients with active JDM. In addition, we measured the sensitivity and specificity of serum neopterin levels in the diagnosis of HLH and evaluated the association of neopterin levels with the laboratory parameters established for the diagnostic criteria.

In this study, the patients diagnosed with HLH had a mean neopterin level that was eight times higher than the cutoff value for normal and four times higher than the levels observed in the control group of patients with active JDM. Neop-
terin levels greater than 38.9 nmol/liter were very specific (95%) and relatively sensitive (70%) for a diagnosis of HLH when the JDM group was used as the comparator. Serum neopterin levels correlated significantly with serum ferritin levels at the time of diagnosis in our HLH patients. Studies have supported that hyperferritinemia, to the extent observed in HLH patients, is a typical characteristic and part of the diagnosis guidelines for HLH. An elevated plasma ferritin level above 500 µg/liter is a criterion for HLH diagnosis (Table 1). It is also suggested that ferritin levels above 10,000 µg/liter appear to be more specific and sensitive for an HLH diagnosis (1). However, there were 2 patients (out of 21 patients) who did not have elevated levels of ferritin at the time of diagnosis. In both patients, neopterin levels were significantly increased (98.5 and 39.7 nmol/liter).

Mutations in PRF1, STX11, and UNC13D are important in the diagnosis of familial HLH, but these mutations occur in only 50% of familial HLH. Soluble IL-2 receptor alpha (sIL-2Rα) levels are relatively sensitive and specific for HLH (1) but are not widely available and could not be compared to neopterin levels in our group since sIL-2Rα levels were not measured in all patients. We have established that neopterin levels are significantly elevated in patients with HLH. Prospective studies are being planned to establish the role of neopterin measurements as a criterion in the diagnosis and management of HLH.

As a retrospective review, this study has limitations, some of which have already been addressed. The control group included only JDM patients, which was the only patient group available that routinely had serum neopterin levels measured as an assessment of their disease activity. Elevated serum neopterin levels have been observed with posttraumatic complications among patients with multiple injuries, viral and bacterial infections, and malignancies (3, 7, 14, 15, 18, 20), and no patients with these complications were included in our study. Therefore, a prospective study should be designed to include other patient populations. We also must acknowledge that our patient sample may not represent all of the patients with HLH/MAS seen at CMH during the defined study period, since the patients were identified retrospectively by extraction of specific ICD-9 codes.

In summary, we show that serum neopterin levels are significantly elevated in patients with HLH compared to normal ranges in healthy controls and levels in patients with active JDM. In comparison with levels in active JDM patients, we observed that levels of serum neopterin of >39.8 nmol/liter in patients suspected of HLH are very specific and sensitive for the diagnosis of HLH. We also demonstrated that the elevation of serum neopterin levels in patients with HLH was often very significant (one patient had levels more than 30 times the normal cutoff level), and although levels correlated with ferritin levels (hyperferritinemia), the neopterin levels in two HLH patients were significantly elevated in the absence of elevated (>500 U/liter) ferritin. This demonstrates that serum neopterin measurements warrant further evaluation as a diagnostic parameter for HLH. In addition to a diagnostic marker, the role of serial neopterin measurements in the management of HLH should be evaluated.

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REFERENCES

1. Allen, C. E., X. Yu, C. A. Kozinetz, and K. L. McClain. 2008. Highly elevated ferritin levels and the diagnosis of hemophagocytic lymphohistiocytosis. Pediatr. Blood Cancer 50:1227–1235.
2. Bohan, A., and J. B. Peter. 1975. Polymyositis and dermatomyositis. N. Engl. J. Med. 292:344–403.
3. Castillo, L., and J. Carcillo. 2009. Secondary hemophagocytic lymphohistiocytosis and severe sepsis/systemic inflammatory response syndrome/macrophage activation syndrome share common intermediate phenotypes on a spectrum of inflammation. Pediatr. Crit. Care Med. 10:387–392.
4. De Benedetti, F., M. De Amici, L. Aramini, N. Ruperto, and A. Martini. 1993. Correlation of serum neopterin concentrations with disease activity in juvenile dermatomyositis. Arch. Dis. Child. 2:232–235.
5. Enders, A., et al. 2006. Lethal hemophagocytic lymphohistiocytosis in Hermansky-Pudlak syndrome type II. Blood 108:81–87.
6. Filipovich, A. H. 2006. Hemophagocytic lymphohistiocytosis and related disorders. Curr. Opin. Allergy Clin. Immunol. 6:410–415.
7. Gulcan, E. M., I. Tirit, A. Anil, E. Adal, and G. Ozbay. 2008. Serum neopterin levels in children with hepatitis-B-related chronic liver disease and its relationship to disease severity. World J. Gastroenterol. 14(44):6840–6843.
8. Henter, J.-J., et al. 2007. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr. Blood Cancer 48(2):124–136.
9. Humber, C., et al. 1984. Immune response-associated production of neopterin. Release from macrophages primarily under control of interferon-gamma. J. Exp. Med. 160:310–316.
10. Imashuku, S., et al. 2002. Low natural killer activity and central nervous system disease as a high-risk prognostic indicator for young patients with hemophagocytic lymphohistiocytosis (HLH). Cancer 94:3023–3031.
11. Imashuku, S., et al. 2001. Requirement for etoposide in the treatment of Epstein-Barr virus-associated hemophagocytic lymphohistiocytosis. J. Clin. Oncol. 19(10):2665–2673.
12. Janka, G. E. 2007. Familial and acquired hemophagocytic lymphohistiocytosis. Eur. J. Pediatr. 166:95–109.
13. Kelly, A., and A. V. Ramanan. 2007. Recognition and management of macrophage activation syndrome in juvenile arthritis. Curr. Opin. Rheumatol. 19:477–481.
14. Mildvan, D., et al. 2005. Serum neopterin, an immune activation marker, independently predicts disease progression in advanced HIV-1 infection. Clin. Infect. Dis. 40:476–479.
15. Prat, C., et al. 2008. Evaluation of procalcitonin, neopterin, C-reactive protein, IL-6 and IL-8 as a diagnostic marker of infection in patients with febrile neutropenia. Leuk. Lymphoma 49:1752–1761.
16. Sawhney, S., P. Woo, and K. J. Murray. 2001. Macrophage activation syndrome: a potentially fatal complication of rheumatic disorders. Arch. Dis. Child. 85:421–426.
17. Shimizu, M., et al. 14 May 2010, posting date. Distinct cytokine profiles of systemic-onset juvenile idiopathic arthritis-associated macrophage activation syndrome with particular emphasis on the role of interleukin-18 in its pathogenesis. Rheumatology. doi:10.1093/rheumatology/keq133.
18. Soucher, R., et al. 2010. Neopterin, a prognostic marker in human malignancies. Cancer Lett. 287:13–22.
19. Sumegi, J., J. Johnson, A. Filipovich, K. Zhan, and R. Marsh. 27 February 2004, posting date. Lymphoproliferative disease, X-linked. GeneReviews. http://www.ncbi.nlm.nih.gov/books/NBK1408/.
20. Tatzber, F., et al. 1991. Elevated serum neopterin levels in atherosclerosis. Atherosclerosis 2–3:203–208.