Evaluation of neopterin as a biomarker for the monitoring of Gaucher disease patients

Cristina Drugan1, Tudor C. Drugan2, Nicolae Miron3, Paula Grigorescu-Sido4, Ioana Nașcu4, Cristina Cătănă1

1Department of Medical Biochemistry, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania, 2Department of Medical Informatics and Biostatistics, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania, 3Department of Clinical Immunology, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania, 4Department of Paediatrics, Paediatric Clinic I, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania

Objectives: Biomarker research is an important area of investigation in Gaucher disease, caused by an inherited deficiency of a lysosomal enzyme, glucocerebrosidase. We evaluated the usefulness of neopterin, as a novel biomarker reflecting chronic inflammation and immune system activation in Gaucher disease and analysed its evolution in response to enzyme replacement therapy (ERT).

Methods: Circulating plasma neopterin levels in 31 patients with non-neuronopathic Gaucher disease were measured before and after the onset of ERT and were compared with those of 18 healthy controls. Plasma chitotriosidase activity was also monitored, as a reference biomarker, against which we evaluated the evolution of neopterin.

Results: Neopterin levels were significantly increased in treatment-naïve patients (mean 11.90 ± 5.82 nM) compared with controls (6.63 ± 5.59 nM, Mann–Whitney U test \(P = 0.001\)), but returned to normal levels (6.92 ± 4.66 nM) following ERT. Investigating the diagnostic value of neopterin by receiver operating characteristic analysis, we found a cut-off value of 7.813 nM that corresponds to an area under the curve of 0.780 and indicates a good discrimination capacity, with a sensitivity of 0.774 and a specificity of 0.778.

Discussion: Our results suggest that measurement of circulating neopterin may be considered as a novel test for the confirmation of diagnosis and monitoring of the efficacy of therapeutic intervention in Gaucher disease. Plasma neopterin levels reflect the global accumulation and activation of Gaucher cells and the extent of chronic immune activation in this disorder.

Conclusion: Neopterin may be an alternative storage cell biomarker in Gaucher disease, especially in chitotriosidase-deficient patients.

Keywords: Gaucher disease, Neopterin, Chitotriosidase, Biomarkers, Macrophage activation

Introduction

Gaucher disease, one of the most prevalent lysosomal storage disorders, is caused by a recessively inherited deficiency of the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45), encoded by the \textit{GBA} gene.\textsuperscript{1,2} This metabolic defect results in a progressive accumulation of undegraded substrate, glucosylceramide, in the lysosomes of macrophages, which in turn evolve into the characteristic glycolipid-laden ‘Gaucher’ cells.\textsuperscript{3} As a multisystem disorder, Gaucher disease is characterized by the accumulation of these activated macrophages in various tissues, particularly the spleen, liver, bone marrow, and lungs. Three clinical types have been delineated, according to the absence (type 1, the non-neuronopathic variant) or the presence and evolution of primary neurological involvement (type 2, the acute neuronopathic form and type 3, the chronic neuronopathic form). Nonetheless, these clinical phenotypes are currently perceived as a belonging to a continuous spectrum, rather than separate entities.\textsuperscript{4,5} Current therapeutic options for patients affected by the non-neuronopathic form of the disease include expensive strategies based on enzyme replacement therapy (ERT) with recombinant macrophage-targeted glucocerebrosidase and substrate reduction therapy (SRT), requiring an early diagnosis and an accurate longitudinal follow-up.\textsuperscript{6–8}

A persistent state of low-grade inflammation has been described in Gaucher disease, mediated by pro-inflammatory cytokines secreted by Gaucher cells.\textsuperscript{9,10} These activated macrophages release a large variety of signal molecules, such as chemokines (pulmonary and activation-regulated chemokine CCL18/PARC),
several interleukins, and macrophage inflammatory proteins. In addition, Gaucher cells are the source of many hydrolytic enzymes, such as tartrate-resistant acid phosphatase, cathepsins, lysozyme, and, most importantly, they secrete chitotriosidase. Some of these proteins have become widely used biomarkers for the assessment of the systemic burden of the disease and for long-term monitoring of its response to therapeutic intervention. The search for appropriate biomarkers has led to the establishment of circulating chitotriosidase and CCL18/PARC as the primary surrogate markers, reflecting macrophage activation and total storage cell burden. Recently, glucosylphosphinosine demonstrated remarkable attributes as a circulating biomarker that is not related to the systemic build-up of storage cells, but results from the alteration of the cata- bolic pathway of glucosylceramide.

Many other potential biomarkers have been proposed for the appropriate follow-up of Gaucher patients, reflecting either the metabolic changes induced by the disease or the accumulation of storage cells. Since a genetic polymorphism of the chitotriosidase gene generates a null variant, preventing its use in about 6% of the population, including patients with Gaucher disease and as several other biomarkers require sophisticated assays that are not readily available in hospital laboratories, there is still a need for alternative biomarkers of disease burden and treatment response in Gaucher disease.

Neopterin belongs to the class of pteridines, synthesized from guanosine triphosphate (GTP), by activated macrophages and dendritic cells, following stimulation by interferon-γ and, indirectly, tumour necrosis factor-α. In human macrophages and dendritic cells, GTP-cyclohydrolase I catalyses the conversion of GTP to 7,8-dihydroneopterin triphosphate, the direct precursor of neopterin. In other human cell types (especially fibroblasts and endothelial cells) or cells from other animal species, this pathway continues with the catalytic intervention of pyruvoyl-tetrahydrobiopterin synthase, leading to the formation of 5,6,7,8-tetrahydrobiopterin (BH₄), an important cofactor for many enzymes (nitric oxide synthase, phenylalanine, and tyrosine hydroxylases).

A constitutive deficiency of pyruvoyl-tetrahydrobiopterin synthase in human macrophages and dendritic cells prevents these further metabolic steps. Therefore, circulating neopterin and its partially reduced derivative, 7,8-dihydroneopterin, are secreted by activated macrophages and dendritic cells. In recent years, neopterin has been increasingly recognized as an independent marker of immune system activation and a predictive marker for cardiovascular risk assessment. High circulating levels were described in viral and parasite infections, allograft rejection episodes, autoimmune or malignant diseases, aging, and neurodegenerative conditions. Since the pathogenesis of Gaucher disease involves an interaction between macrophage activation and chronic immune stimulation, a situation in which increased neopterin concentrations have already been described, we conducted this study in order to investigate the informative capacity of this biomarker and its response to long-term ERT in type 1 Gaucher disease patients.

Materials and methods

Patients and control subjects

The study comprised 31 adult patients with type 1 Gaucher disease (15 males and 16 females, mean age 38.03 years), in whom the diagnosis had been confirmed by measurement of glucocerebrosidase activity in peripheral blood leukocytes. All of them were treated with ERT, receiving individualized doses of imiglucerase (Sanofi-Genzyme) ranging from 28 to 60 U/kg, once every two weeks, during an average interval of 3.8 years (range 3–110 months). Clinical evaluation and follow-up were performed at the Centre for genetic disorders of the University of Medicine and Pharmacy, Cluj-Napoca, Romania.

The control group consisted of 18 healthy volunteers (16 males and 2 females), with a mean age of 58.89 years. All patients and controls signed an informed written consent and the use of their peripheral blood samples was approved by the Ethics Committee of the University of Medicine and Pharmacy (Cluj-Napoca, Romania).

Sample collection

All blood samples were centrifuged within less than one hour and stored at −20°C. Patients were selected on the basis of the availability of stored frozen plasma samples, collected at the moment of diagnosis, before the initiation of ERT. Subsequent patient samples were collected during routine blood work-ups and were subjected to the same procedure. Control blood samples provided by healthy volunteers were processed and stored identically.

Measurement of plasma chitotriosidase activity

Plasma chitotriosidase activity was determined according to the standard enzymatic assay, using the fluorescent substrate 4-methyl-umbelliferyl-β-D-N,N′,N″-triacetylchitotrioside.

Measurement of plasma neopterin concentration

Plasma neopterin levels were measured by a sandwich enzyme-linked immunosorbent assay using a commercially available kit (www.antibodies-online.com), according to the recommendations of the

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manufacturer. Concentrations were calculated using a linear regression equation and were expressed in nanomoles/litre (nM).

**Mutation analysis in Gaucher disease patients**
The analysis of frequent mutations in the glucocerebrosidase gene (N370S, L444P, 84GG, and R463C) was performed by polymerase chain reaction (PCR) amplification and restriction enzyme digestion, according to previously described methods.33–35 The complex alleles resulting from recombination events between the glucocerebrosidase gene and its pseudogene, designated recTL (including mutations D409H, L444P, A456P, and V460V), recNciI (associating mutations L444P, A456P, and V460V), and recA456P (associating mutations L444P and A456P) were analysed by direct sequencing of the amplified fragments harbouring the L444P substitution.

**Statistical analysis**
Data analysis was performed using the IBM SPSS (version 22.0) and SigmaPlot (version 12.00) software. Quantitative variables were presented as mean ± standard deviation (SD). Correlations were analysed using the Pearson coefficient and its significance test. Normality was tested with the Shapiro–Wilk test. Because biomarker values did not display a normal distribution (Shapiro–Wilk test probabilities were between 0.000 and 0.032, for patient and control groups alike), independent sample distributions were compared with the non-parametric Mann–Whitney U test or the Kruskal–Wallis analysis of variance test for multiple groups. Receiver operating characteristic (ROC) analysis was used to establish the optimal cut-off value of plasma neopterin that corresponds to the best association between sensitivity and specificity. For this analysis we used the specific toolbox module from the SigmaPlot software. Results were considered significant if the value of \( p \) was less than 0.05.

**Results**

**Plasma neopterin concentrations in untreated Gaucher patients and in control subjects**
To evaluate the potential value of neopterin as a biochemical marker of Gaucher disease, we measured its concentration in plasma samples from patient and control groups. We also measured plasma chitotriosidase activity for a comparative analysis with a well-established biomarker of Gaucher disease.

The patient group comprised 31 patients with type 1 Gaucher disease, diagnosed on the basis of clinical signs, deficient glucocerebrosidase activity, and molecular genetic analysis. All of them were classified as having the non-neuronopathic form of the disease, based on the absence of neurological involvement. All patients declared a Caucasian, non-Jewish ethnicity.

Measurement of plasma neopterin concentrations revealed major differences between the untreated patients and the control subjects (Fig. 1). Mean plasma neopterin value was significantly increased in Gaucher patients (11.90 ± 5.82 nM), compared to the controls (6.63 ± 5.59 nM, Mann–Whitney U test \( P = 0.001 \)). Similarly, mean plasma chitotriosidase activity was markedly increased in Gaucher patients, compared to the control subjects (35 460 ± 28 058 nmol/mL/h versus 582 ± 501 nmol/mL/h, Mann–Whitney U test \( P < 0.001 \)).

**Influence of ERT on plasma neopterin levels in Gaucher patients**
To investigate the usefulness of circulating neopterin as a biochemical marker reflecting the changes induced by ERT, we measured its concentration in plasma samples taken after several years of regular infusions and compared it to pre-therapeutic levels. A significant decrease in plasma neopterin concentration was observed (Fig. 1A) and, as expected, a striking decline in plasma chitotriosidase activity was also noted (Fig. 1B).

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**Figure 1** (A) Mean neopterin (nM) and (B) chitotriosidase (nmol/mL/h) values in Gaucher disease patients and control subjects and their variation following ERT.
Mean neopterin levels in treatment-naïve patients were significantly higher than those measured following ERT (11.90 ± 5.82 nM versus 6.92 ± 4.66 nM, Mann–Whitney U test P < 0.001. However, no significant differences were found between mean neopterin levels after several years of treatment and those measured in control subjects (6.92 ± 4.66 nM versus 6.63 ± 5.59 nM, Mann–Whitney U test P = 0.671). Chitotriosidase evolution followed a similar pattern, with mean pre-therapeutic activity significantly higher than that observed after ERT (35.460 ± 28.058 nmol/mL/h versus 11.254 ± 15.093 nmol/mL/h, Mann–Whitney U test P < 0.001), but its decline did not reach the mean value observed in healthy controls (11.254 ± 15.093 nmol/mL/h versus 582 ± 501 nmol/mL/h (Mann–Whitney U test P < 0.001).

We found no correlation between neopterin and chitotriosidase values in controls (Pearson correlation coefficient r = 0.214, P = 0.393) or in Gaucher disease patients, either before (r = 0.011, P = 0.955) or after the initiation of ERT (r = 0.078, P = 0.675).

Relationship between biomarker concentrations and the most frequent genotypes
In order to investigate a possible relationship between the level of circulating neopterin and the biological consequences of mutations in the glucocerebrosidase gene, we analysed the most frequent mutations in Gaucher disease patients. This allowed the characterization of the following genotypes: N370S/unknown allele (14 patients, 45.20% of total), N370S/L444P (9 patients, 29.00%), and N370S/N370S (5 patients, 16.10%). Less frequent genotypes (N370S/R463C, N370S/recA456P, and unknown allele/unknown allele) were each present in only one patient (3.20% for each genotype) and, as such, were not included in the statistical analysis.

The variations of mean plasma neopterin concentration and of mean plasma chitotriosidase activity were analysed as a function of the most frequent genotypes identified in our patients. Table 1 synthesizes the results of the statistical analyses, for baseline (pre-ERT) and post-ERT values: the horizontal comparisons with the Kruskal–Wallis test revealed no significant differences between the mean values of either neopterin, or chitotriosidase, corresponding to the different genotypes.

The variations of mean neonopterin level and of mean chitotriosidase activity for the most frequent genotypes, as a function of treatment status, were evaluated with the Mann–Whitney U test. These results are vertically displayed in Table 1. For the genotypes N370S/unknown allele and N370S/L444P, the variations were statistically significant, for both neopterin and chitotriosidase. For the genotype N370S/N370S, even if an arithmetic difference was noted for mean chitotriosidase activity, we found no significant variations, for both neopterin and chitotriosidase, according to the Mann–Whitney U test. These results are illustrated by Fig. 2 for neopterin and Fig. 3 for chitotriosidase.

Assessment of the diagnostic value of neopterin ROC analysis was carried out to assess the discriminating capacity of neopterin, in comparison with chitotriosidase (Fig. 4). Both curves are describing accurate diagnostic tests, with an area under the curve (AUC) and confidence intervals (CI) of 0.780 (0.635–0.924) for neopterin and 1.000 (1.000–1.000) for chitotriosidase (Table 2). Our results indicate a good diagnostic capacity for neopterin, with an optimal performance in discriminating between Gaucher patients and controls at values above 7.613 nM, corresponding to a sensitivity of 0.774 and a specificity of 0.778.

Discussion
The involvement of the immune system in the pathogenesis of Gaucher disease has been increasingly recognized as a key factor in the clinical expression of this complex disease. The accumulation of glucosylceramide may be the trigger that initiates a systemic response.

![Table 1 Mean plasma neopterin and chitotriosidase variation as a function of genotype and treatment status](image-url)
inflammatory reaction, characterized by macrophage activation and metabolic alterations. As an ideal biomarker is not only expected to provide insight into the disease burden, but also to reflect the pathophysiology of the disease, we chose to investigate the attributes of neopterin, as a biochemical marker that may combine clinical monitoring of disease severity with information on the extent of chronic immune activation. Indeed, elevated plasma neopterin levels have been reported in numerous inflammatory conditions associated with macrophage activation and, as such, its circulating level may reflect the global accumulation and persistent activation of Gaucher cells.

A previous study by Casal et al. reported increased serum levels of macrophage activation markers, including neopterin, in type 1 Gaucher disease patients. Their research focused on a comparative analysis of several biomarkers and documented their variation in response to ERT. We further analysed the properties of neopterin and revealed its usefulness as a biomarker of Gaucher disease. We observed a significant increase in plasma neopterin levels in untreated symptomatic patients, compared to the normal controls. Furthermore, in our patients, long-term ERT has led to the normalization of mean neopterin concentration, in contrast to the evolution of chitotriosidase, which did not reach normal levels, even after several years of treatment. On the other hand, there was no correlation between chitotriosidase activity and neopterin concentration. Taken together, these findings suggest that neopterin and chitotriosidase secretion may diverge at a certain point during the process of macrophage activation. Moreover, the normalization of plasma neopterin concentration after long-term ERT suggests an attenuation of the inflammatory response, in contrast to other, more refractory, manifestations of the disease, especially skeletal lesions, whose persistence was probably reflected by higher chitotriosidase activity.
We analysed the distribution of mean neopterin and chitotriosidase values according to the most frequent genotypes identified in our patients. There was no significant difference for either biomarker, but, in the case of neopterin, we observed higher concentrations in untreated patients carrying the L444P allele or an unidentified mutation, compared to those who were homozygous for the milder allele, N370S. This suggests that circulating neopterin levels may be related to the pathogenic events that lead to the clinical manifestations of the disease, but, taking into account the limited number of patients analysed for each genotype, further studies are mandatory.

Following the initiation of ERT, the levels of neopterin and of chitotriosidase displayed a significant decrease in patients affected by the genotypes N370S/unknown allele and N370S/L444P, compared to the patients who were homozygous for the N370S allele. A less extensive biomarker decline in this patient group may be the consequence of a milder phenotypic expression of the disease, and, at least in the case of neopterin, may suggest a lower degree of tissue inflammation.

To test the ability of neopterin to accurately discriminate between Gaucher disease patients and normal controls, we performed the ROC analysis. Although in our patient cohort chitotriosidase performs better as a discrimination biomarker (AUC 1.000, sensitivity 0.968, and specificity 1.000), neopterin can still be considered an attractive diagnostic test at levels above the cut-off value of 7.613 nM, corresponding to a good degree of sensitivity and specificity.

Our findings suggest that circulating neopterin may be included in the biomarker panel and its measurement may be considered useful for the confirmation of diagnosis and monitoring of the efficacy of therapeutic intervention in Gaucher disease patients. Our results also indicate a more spectacular increase in chitotriosidase activity in symptomatic patients and a better discrimination capacity of this biomarker between patient and control groups, but the major disadvantage of chitotriosidase is the high prevalence of its complete deficiency, affecting Gaucher patients who are homozygous for an insertion polymorphism in its gene and distorting the results of long-term monitoring in patients who are heterozygous for this genetic variant.26 In such patients, neopterin could serve as an interesting alternative biomarker.

There is accumulating evidence about the involvement of neopterin and its derivative, 7,8-dihydronoeopterin, in modulating the release and cytotoxicity of reactive oxygen species by activated macrophages.30 Higher neopterin concentrations were reported in association with reduced levels of serum antioxidant compounds and vitamins, probably as a result of their rapid consumption.41 Measurement of neopterin levels may provide valuable information about the extent of oxidative stress associated with chronic immune activation. In this context, the analysis of neopterin secretion by activated Gaucher cells could open new research avenues to the pathogenesis of Gaucher disease.

The limitations of our study are related to the lack of serial neopterin measurements, required for the exploration of its relationship with disease manifestations and evolution. Further studies documenting the correlations between neopterin levels and clinical observations, including the response to long-term ERT or SRT are mandatory. Additionally, the implications of elevated neopterin levels in the pathophysiology of Gaucher disease deserve further investigation.

In conclusion, circulating neopterin may be considered a reliable biomarker for Gaucher disease, especially in chitotriosidase-deficient patients. An advantage of neopterin measurement is related to its accurate monitoring by inexpensive techniques, readily available in hospital laboratories. As a storage cell biomarker, plasma neopterin concentration reflects the global accumulation and activation of Gaucher cells and provides valuable information on the extent of chronic immune activation in this complex disorder.

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Table 2 ROC curve parameters for neopterin and chitotriosidase

| Biomarker | AUC | Standard error | 95% CI | P value |
|-----------|-----|---------------|------|---------|
| Neopterin | 0.780 | 0.074 | 0.635–0.924 | <0.0001 |
| Chitotriosidase | 1.000 | 0.000 | 1.000–1.000 | <0.0001 |

Sensitivity (95% CI) for neopterin: 0.774 (0.589–0.904) and for chitotriosidase: 0.778 (0.524–0.936).

Specificity (95% CI) for neopterin: 0.778 (0.524–0.936) and for chitotriosidase: 0.774 (0.589–0.904).

Cut-off (nmol/mL/h) for neopterin: 7.613 and for chitotriosidase: 6.000 nmol/mL/h.
and analysis tools contribution. P. Grigorescu-Sido: clinical diagnosis and patient follow-up, collection of clinical data. I. Nasceu: clinical diagnosis and patient follow-up, collection of clinical data. C. Catana: study design, experimental analysis and equipment and analysis tools contribution. The final version of the manuscript has been read and approved by all authors.

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Conflict of interest The authors declare no conflict of interest related to the present study.

Ethics approval All patients and controls signed an informed written consent and the use of their peripheral blood samples was approved by the Ethics Committee of the University of Medicine and Pharmacy (Cluj-Napoca, Romania).

ORCID Cristina Drugan http://orcid.org/0000-0001-9136-2350 Tudor C. Drugan http://orcid.org/0000-0003-0097-262X

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