α_1 -Acid glycoprotein as a putative biomarker for monitoring the development of the type II reactional stage of leprosy

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Leprosy, a spectral disease manifested on the basis of host immune responses, is complicated by its reactional stages, namely type I reversal reaction (RR) and type II erythema nodosum leprosum (ENL). These reactional stages are characterized by uncontrolled and aberrant immune responses. Biomarkers for reactional stages would aid in early diagnosis, efficient treatment, prevention of neurological complications and prediction of predisposition to reactional stages. In this study, comparative analysis of the serum proteome of leprosy patients by two-dimensional electrophoresis (2DE) followed by mass spectrometry showed differential expression of acute-phase protein α_1 -acid glycoprotein (AGP; also known as orosomucoid). AGP levels in untreated ENL cases were significantly higher than in lepromatous leprosy (LL; a non-reactional disease stage) ($P=0.0126$), RR ($P=0.0176$) and healthy controls ($P=0.0030$). These data were confirmed using ELISA. The levels of AGP decreased to normal levels after treatment with multidrug therapy and thalidomide ($P=0.0167$). In a follow-up study, AGP levels, which were high in the untreated ENL stage, decreased significantly at 5 days ($P=0.0084$) and 21 days ($P=0.0027$) post-treatment. A stage-dependent increase in AGP in an LL patient who progressed into the ENL stage was also shown. Glycosylation analysis by 2DE showed differential expression of acidic glycoforms of AGP in untreated ENL cases. Changes in AGP concentration and differential expression of isoforms correlated with the inflammatory condition in ENL and also with the treatment regimen. Thus, initial validation of AGP as an ENL-specific biomarker and treatment indicator was shown in this study.

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INTRODUCTION

Leprosy, a chronic infectious disease caused by *Mycobacterium leprae*, affects the skin and peripheral nerves. Leprosy elimination efforts have reduced the prevalence of active cases; however, the incidence levels in endemic countries have not been significantly altered, indicating ongoing transmission of the disease (Lockwood, 2002;

Richardus & Habbema, 2007). Development of highly specific and sensitive diagnostic methods to screen this population is currently needed. As the genomes of various *M. leprae* clinical isolates are highly conserved (Monot *et al.*, 2005) and only a fraction of exposed individuals develop late stages of the disease (Steger & Barrett, 1994), it is likely that host–pathogen interactions and consequent host physiological and immunological alterations will be significant in understanding progression of the disease.

Leprosy presents as a spectrum of conditions, ranging from the paucibacillary tuberculoid leprosy (TT) stage to the multibacillary lepromatous leprosy (LL) stage with intermediate borderline forms (Ridley & Jopling, 1966). These intermediate borderline forms are unstable, leading to reactional conditions manifested in the form of reversal reactions (RR; type 1 reaction) and erythema nodosum leprosum (ENL; type 2 reaction). RR occurring in borderline patients is characterized by an augmented cell-mediated immune response against the antigens of *M. leprae* (Ridley, 1988; Steger & Barrett, 1994). ENL occurs in

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Abbreviations: 2DE, two-dimensional electrophoresis; AGP, α_1 -acid glycoprotein; BB, mid-borderline; BL, borderline lepromatous; BT, borderline tuberculoid; ENL, erythema nodosum leprosum; IPG, immobilized pH gradient; LL, lepromatous leprosy; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MDT, multidrug therapy; RR, reversal reaction; sLe^x, sialyl Lewis x; TNF- α , tumour necrosis factor- α ; TT, tuberculoid leprosy.

Clinical information on the leprosy patients in this study and details of MALDI-TOF MS identification of α_1 -acid glycoprotein are available with the online version of this paper.

borderline (BL) as well as LL patients. Deposition of *M. leprae* antigen–antibody immune complexes in the vessels, followed by neutrophil infiltration and complement activation, lead to systemic inflammatory responses in ENL (Kahawita & Lockwood, 2008).

Systemic responses in leprosy also reflect the localized responses seen in peripheral lesions (Salgame *et al.*, 1991; Yamamura *et al.*, 1991; Misra *et al.*, 1995). *M. leprae*-specific phenolic glycolipid-1 antigen-based assays have been studied extensively for their application in the diagnosis of the leprosy spectrum and reactions, monitoring of treatment, prediction of relapse and screening of contacts as well as the general population (Young & Buchanan, 1983; Oskam *et al.*, 2003).

Host responses, as reflected in the serum proteome profile, could be an early indication of disease progression. Considering the morbidity and systemic inflammation associated with ENL conditions, identification of serum biomarkers will be of prognostic value. High-resolution two-dimensional electrophoresis (2DE) of plasma proteins is an important tool in parallel analysis of all proteins, their changes relative to each other and their post-translational modifications. A previous study by us involving 2DE proteome profiles of serum from leprosy patients showed differential expression of the acute-phase protein haptoglobin isoform specifically in ENL patients (Gupta *et al.*, 2007).

In this study, we have reported differential expression and glycosylation of another acute-phase protein, α_1 -acid glycoprotein (AGP), in ENL cases by 2DE and ELISA. The effect of drug treatment on AGP levels and glycan pattern is also shown.

METHODS

Serum samples. Serum samples were collected from healthy controls ($n=9$) in Madurai Kamaraj University, Madurai, India, and from leprosy patients ($n=54$; see Supplementary Table S1, available in JMM Online) at the Voluntary Health Services Centre, Sakthi Nagar, Erode, Tamil Nadu, India. Patients were classified immunohistologically into five groups according to the Ridley and Jopling system of classification (Ridley & Jopling, 1966): polar tuberculoid (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL) and lepromatous (LL). Leprosy patients undergoing RR and ENL reactions were also recruited in this study. Patients with exaggeration and swelling of existing skin lesions alongside neuritis were categorized as RR. Clinical features of ENL patients included painful and tender nodules, joint pain, fever, iridocyclitis, long-term nephritis, orchitis and bone pain. Under the exclusion criteria, the patients were routinely screened for human immunodeficiency virus infection, tuberculosis infection and minor ailments to rule out proteome profile changes as a result of other causes. Patient sera were collected with their consent and following the rules prescribed by the Institutional Ethical Committee and Indian Council of Medical Research (<http://icmr.nic.in>).

Serum collection. Blood samples were collected by venipuncture into glass tubes and allowed to clot for 30 min at room temperature. Serum was then separated from the clot by centrifugation at 1300 g

for 10 min. Stock serum was stored in liquid nitrogen and smaller aliquots (100 or 50 μ l) were stored at -70°C until further use.

Sample preparation. Highly abundant proteins, albumin and IgG were depleted from the serum prior to 2D-PAGE analysis. Sixty microlitres of serum sample was treated with an Aurum Serum Protein Mini kit (Bio-Rad) as described by Gupta *et al.* (2007). The efficiency of depletion was first checked by one-dimensional SDS-PAGE as described previously (Gupta *et al.*, 2007). The depleted serum was then subjected to 2DE.

2DE of serum proteins. Protein levels were estimated using the method of Bradford (1976). Serum samples were solubilized in rehydration buffer (Gupta *et al.*, 2007) followed by passive rehydration of immobilized pH gradient (IPG) strips (GE Healthcare) for a minimum of 12 h. Focusing at 20°C was carried out using the IPGphor IEF apparatus (GE Healthcare) as follows: 500 V for 1 h (step and hold), 1000 V for 1 h (gradient), 8000 V for 3 h (gradient) and 8000 V for 5 h (step and hold). For active rehydration (carried out for narrow-range 3.5–4.5 NL 18 cm IPG strips), rehydration at 30 V for 11 h was followed by isoelectric focusing: 300 V for 2 h (step and hold), 1000 V for 1 h (gradient), 8000 V for 1.5 h (gradient) and 8000 V for 9 h (step and hold). Electrophoresed strips were subjected to a two-step equilibration followed by second-dimension electrophoresis as described previously (Gupta *et al.*, 2007).

Staining. Gels were stained using colloidal Coomassie blue (Candiano *et al.*, 2004) or a matrix-assisted laser desorption/ionization (MALDI)-compatible silver staining method as described previously (Mortz *et al.*, 2001).

Image analysis. Image analysis of gels was carried out using ImageMaster 2D Platinum version 5.0 software (GE Healthcare). For comparisons of differential expression, all control and patient 2D gels were assigned to two different classes. Spots were detected using the default setting of the software and then matched between each gel and the reference gel. Visual inspection was used to confirm the data points where needed. The quantification parameter used for comparison was percentage intensity, which referred to the intensity of the protein spot against the total intensity of all spots in the gel. The statistical significance of the differences between the healthy controls and the patients was determined using Student's *t*-test.

In-gel tryptic digestion and MALDI-time of flight (TOF) MS analysis. Stained protein spots were cut out and subjected to tryptic digestion, concentration and desalting using established procedures as described previously (Gupta *et al.*, 2007). Peptide samples in α -cyano-4-hydroxycinnamic acid matrix were applied to a stainless steel MALDI target plate using the sandwich method. A peptide mass spectrum was acquired using an Axima CFR plus (KRATOS Shimadzu) MALDI-TOF mass spectrometer in the reflectron mode. Instrument calibration and spectrum acquisition details were as described by Gupta *et al.* (2007). The NCBIInr database in MASCOT version 2.2 (www.matrixscience.com) was searched using the following parameters: tolerance, 0.05–1 Da; species, *Homo sapiens*; missed cleavages, 1; fixed modification, carbamidomethyl; variable modifications, oxidation of methionine and propionamide. Proteins with a significant score ($P < 0.05$) were confirmed as identified.

Quantitative ELISA. Sandwich ELISA was performed using a Human AGP ELISA Quantification kit (Genway Biotech) according to the manufacturer's instructions. Three dilutions (1:2000–1:4000) were used for each serum sample and the mean reading was determined. The statistical significance was determined using Student's *t*-test.

RESULTS AND DISCUSSION

Analysis of AGP expression in ENL patients by 2DE

Rapid serological diagnostic tests together with rigorous epidemiological surveys may help in early diagnosis and in breaking the transmission chain in leprosy. Various assays that detect leprosy-specific antibody responses have been developed, such as ELISAs (Young & Buchanan, 1983), an *M. leprae* gelatin particle agglutination test (Izumi *et al.*, 1990), an *M. leprae* dipstick test (Bühner-Sékula *et al.*, 2000) and an *M. leprae* lateral flow test (Bühner-Sékula *et al.*, 2003). These have helped in identifying subclinical infection in contact populations, identification of misclassification of multibacillary patients, classification of the leprosy spectrum and monitoring of treatment (Oskam *et al.*, 2003). However, antibody levels do not correlate well with paucibacillary leprosy stage and reactional conditions. Thus, sensitive serological markers are required to detect clinical as well as subclinical conditions. Also, as reactions are the major cause of nerve damage in leprosy, serological

tests for prediction of reactions are in great demand (Oskam *et al.*, 2003).

Serum proteome analysis (Fig. 1a) showed upregulation of a highly acidic protein in the samples from ENL patients, and the increase in expression was significant. Multiple samples were analysed to confirm these results (Fig. 1b). 2DE data were confirmed using Western blot analysis (data not shown). MALDI-TOF MS identified this protein as AGP, also known as orosomucoid (Fig. 1c and Supplementary Fig. S1, available in JMM Online). Image analysis showed an intensity of $1.865 \pm 0.296\%$ (mean $\pm$ SD) in controls and $4.108 \pm 0.676\%$ in ENL cases (Fig. 1d). This twofold increase was observed in all ENL patients, and the data were statistically significant ($P < 0.0001$). In one of the patients, a fourfold decrease was observed during the combined multidrug therapy (MDT) and thalidomide treatment compared with untreated AGP levels (data not shown).

AGP is an immunomodulatory acute-phase protein with pro-inflammatory as well as anti-inflammatory properties; in addition, AGP binds to several commonly used drugs (Van Dijk *et al.*, 1998; Hochepped *et al.*, 2003; Silva

Fig. 1. Relative levels of AGP in healthy controls and ENL cases. (a) Representative serum proteome profile from an ENL patient. Neat serum protein (300 μ g) was loaded for IEF using 4–7 pI 18 cm strips and the 2D gels were stained with colloidal Coomassie. (b) Partial view of the 2D gels showing AGP spots from healthy controls ($n=6$) and ENL patients ($n=6$). Whole serum protein (200 μ g) was loaded for IEF using pH 3–10 NL strips (11 cm) and 12.5% gels were used for the second-dimension run, followed by colloidal Coomassie staining. (c) MALDI-TOF identification of AGP (orosomucoid). (d) Histogram showing the percentage intensity of the AGP spot in controls ($n=6$) and ENL patients ($n=6$). The percentage intensity was calculated using a partial view of all 2D gels containing approximately 50 spots. Results are shown as means $\pm$ SD. The difference was statistically significant ($P < 0.0001$).

Miranda *et al.*, 2006; Ceciliani & Pocacqua, 2007). Its major biological functions include inhibitory effects on neutrophil activation, chemotaxis and oxidative metabolism; platelet aggregation; and lymphocyte proliferation (Hochepied *et al.*, 2003). AGP also has antibacterial and anti-apoptotic properties. In addition to these functions, AGP acts as a non-specific competitor for cell surfaces, interfering with host cell–microbe interactions and thus protecting against infections (Ceciliani & Pocacqua, 2007). An increase in serum AGP levels and its altered glycosylation have been shown in various inflammatory disorders and cancers (Ceciliani & Pocacqua, 2007). An increase in AGP concentrations in the urine and tissue samples of patients with invasive urinary bladder cancer (Irmak *et al.*, 2005) and diabetic kidney disorder (Jain *et al.*, 2005) has been shown by 2DE analysis.

ELISA-based quantification of AGP levels

AGP levels in patients across the leprosy spectrum. The ELISA data shown in Fig. 2(a) confirmed the elevated levels of AGP in ENL cases. However, the levels decreased significantly ($P=0.0167$) in MDT- and thalidomide-treated ENL patients and were comparable to the control levels (Fig. 2a). AGP levels in healthy controls, taken as the reference levels in this study, were in the range of $0.05\text{--}0.13\text{ mg ml}^{-1}$, which is lower than the normal AGP levels of $0.5\text{--}1\text{ mg ml}^{-1}$ reported in sera of Western populations (Ceciliani & Pocacqua, 2007). Lower serum AGP concentrations in Indians have been reported previously (Johnson *et al.*, 2004). AGP levels in untreated ENL patients were significantly higher than those in LL ($P=0.0126$) and RR ($P=0.0176$) patients and controls ($P=0.0030$). However, interestingly, the AGP level was much higher in unstable borderline cases, similar to ENL cases (BT, $P=0.0982$; BB, $P=0.5043$; BL, $P=0.1569$). It is possible that these cases could be predisposed to progress to the ENL stage, but additional studies are needed to confirm this.

Thus, our data clearly showed that increased AGP concentrations could be a potential ENL biomarker, distinguishing it from LL, RR and healthy controls. The borderline cases that showed high serum AGP levels could be monitored for ENL development. Previous studies have shown an increase in β_2 -microglobulin (Saha *et al.*, 1985), lactoferrin (Parkash *et al.*, 1993), C-reactive protein (Silva *et al.*, 2007), neopterin (Hamerlinck *et al.*, 1999; Faber *et al.*, 2004), adenosine deaminase (Nigam *et al.*, 2005), chitotriosidase (Iyer *et al.*, 2009), gamma interferon and soluble interleukin-6R (Iyer *et al.*, 2007) in the serum of ENL patients. Most of these markers were also shown to respond to drug treatment; therefore, in our study we also monitored the levels of AGP during treatment.

Effect of treatment on AGP levels in leprosy patients.

Leprosy reactions can occur before chemotherapy, during the course of chemotherapy or even after completion of anti-leprosy therapy (Girdhar *et al.*, 2007). LL patients progress to

ENL even during the treatment period, and we examined the AGP levels in one such patient. AGP levels were quantified in the untreated stage in this LL patient on days 1 and 5 after

Fig. 2. AGP levels across the leprosy spectrum quantified by ELISA. (a) Scatter plot depicting AGP levels in sera from controls ($n=9$) and BT ($n=5$), RR ($n=7$), BB ($n=4$), BL ($n=5$), LL ($n=6$), ENL untreated (UT; $n=6$) and ENL treated (T; $n=6$) patients quantified by sandwich ELISA. **, $P < 0.01$; *, $P < 0.05$; NS, not significant. (b) AGP levels in an LL patient progressing into the ENL stage (see Results). (c) AGP levels in ENL patients at different stages of treatment: day 1 of ENL (untreated) and days 5 and 21 post-treatment.

MDT treatment. Nearly 2 months after MDT treatment, the patient developed ENL. The serum sample collected on the day that ENL was diagnosed clearly showed a fourfold increase, which returned to normal levels after 5 and 21 days of thalidomide and MDT treatment (Fig. 2b).

Additional ENL patients were followed from the day of diagnosis to 5 and 21 days post-treatment. AGP levels, which were high in the untreated ENL stage, had decreased significantly at 5 days ($P=0.0084$) and 21 days ($P=0.0027$) post-treatment (Fig. 2c). Thus, AGP levels responded to drug treatment as early as 5 days and could be a potential marker for determining treatment efficacy. AGP has been used as a marker for treatment response in Crohn's disease, an inflammatory bowel disease that exists in two forms, with respect to cytokine profiles (Gilberts *et al.*, 1994), analogous to lepromatous and tuberculoid conditions in leprosy. It has also been shown that, within 1 month of anti-tumour necrosis factor (TNF)- α therapy for Crohn's disease patients, AGP levels decrease, suggesting clinical improvement (Kupcova *et al.*, 2003).

Interestingly, AGP has been shown to interact with thalidomide (Turk *et al.*, 1996), a drug used in ENL treatment. Thalidomide, an immunomodulatory drug, rapidly ameliorates the inflammatory condition in ENL (Theophilus, 1980) and decreases TNF- α secretion by more than 90 % (Barnes *et al.*, 1992). *In vitro* studies have shown that AGP modulates LPS-induced secretion of the cytokines interleukin-1 β , interleukin-6 and TNF- α by monocytes and macrophages (Boutten *et al.*, 1992). Further AGP-induced TNF- α production has been shown to be enhanced by binding to serum proteins such as complement factor C3 and IgG (Su *et al.*, 1999), both of which are known to increase in the serum of ENL patients (Kahawita & Lockwood, 2008). Therefore, increased AGP concentrations

could result in increased pro-inflammatory cytokines and could act as a precipitating factor in the pathogenesis of ENL.

Glycosylation pattern of AGP in leprosy

AGP has a carbohydrate content of 45 % attached in the form of five *N*-linked glycosylation sites (Hochepied *et al.*, 2003). The relative amount of the different AGP glycoforms in plasma is known to be variable and dependent on pathophysiological conditions and the state of inflammation (Van Dijk *et al.*, 1998). The initial experimental conditions used in this study (IEF using a broad pI range of 4–7) failed to resolve the glycoforms. First-dimension IEF was then carried out using narrow-range IEF strips to resolve the glycoforms. In addition, the same sample was analysed using pI strips with ranges of 4–7, 3–5.6 and 3.5–4.5, followed by second-dimension electrophoresis (Fig. 3).

The serum samples that showed upregulation of AGP in ENL patients were again subjected to IEF using narrow-range IEF strips. IEF on narrow-range strips (Fig. 3c) revealed the presence of highly acidic glycoforms of AGP (Fig. 3c, row i, solid black circle), which decreased post-treatment (Fig. 3c, row ii, dashed circle) and in ENL patients who recovered from the reaction (Fig. 3c, row iii, dotted circle). These glycoforms were, however, observed as a single upregulated spot of AGP on a broad-range IEF strip (compare Fig. 3a and b, row i, with Fig. 3c, row i).

Fig. 4 compares the glycoforms of AGP in healthy controls and ENL patients at different stages of treatment. These acidic glycoforms, observed in untreated ENL samples (Fig. 4b and c, solid black circle), were not observed in control samples (Fig. 4a). Also, these glycoforms responded to treatment in ENL patients (Fig. 4b and c, dashed circles) by decreasing to the levels observed in controls.

Fig. 3. Effect of pI on separation of AGP spots in 2D gels. Partial views of a 2D gel showing the AGP spot for an ENL sample are depicted: (a) 300 μ g neat serum protein, 4–7 pI, with colloidal Coomassie staining; (b) 300 μ g neat serum protein, 3–5.6 NL pI, with colloidal Coomassie staining; (c) 50 μ g albumin- and IgG-depleted serum protein, 3.5–4.5 NL pI, with silver staining. Row (i) represents untreated ENL serum, row (ii) after 18 months of MDT and thalidomide treatment, and row (iii) after recovery from ENL, BL stage.

Fig. 4. AGP glycoforms in ENL follow-up cases and controls. Albumin- and IgG-depleted serum protein (50 μ g) was separated using 3.5–4.5 NL pl 18 cm IPG strips and the 2D gels were silver-stained. A partial view of the 2D gels is given, showing the 45 kDa region with AGP glycoforms for controls (a) and ENL cases (b, c).

A differential glycosylation pattern of AGP has been reported in various diseases such as rheumatoid arthritis, systemic lupus erythematosus, liver cirrhosis, asthma, type 1 diabetes and familial Mediterranean fever (Fournier *et al.*, 2000; Ceciliani & Pocacqua, 2007). Use of narrow-range IEF followed by 2DE can resolve the glycoforms of AGP (Kleinert *et al.*, 2007). In this study, highly acidic glycoforms (with a higher content of sialic acid) with slightly increased molecular mass (representing a high degree of glycan branching; i.e. tri- and tetra-antennary types of glycans) have been found in untreated ENL patients. These glycoforms are absent in healthy controls as well as in treated ENL patients. Lacunza *et al.* (2007) showed an increase in the acidic glycoforms of AGP by capillary IEF in ovarian cancer and lymphoma patients when compared with healthy controls. The tri- and tetra-antennary oligosaccharides included fucose residues that form the sialyl Lewis x (sLe^x) structures (Ceciliani & Pocacqua, 2007). The increased levels of sLe^x-bearing AGP glycoforms may affect the interaction between endothelial E-selectin and sLe^x-bearing leukocytes by competing with leukocytes for E-selectins. This competition inhibits the extravasation

of the granulocytes into inflamed tissues (De Graaf *et al.*, 1993). Interestingly, tri- and tetra-antennary types of glycan act as suppressors of lymphocyte proliferation and TNF- α production (Ceciliani & Pocacqua, 2007), both factors involved in immunopathology of ENL (Kahawita & Lockwood, 2008). Thus, the increase in acidic glycoforms in untreated ENL cases in this study is suggestive of an anti-inflammatory role of increased levels of AGP. Increases in branched glycans and highly fucosylated sLe^x AGP forms reported in various inflammatory diseases such as rheumatoid arthritis, hypergammaglobulinaemia D and familial Mediterranean fever (Ceciliani & Pocacqua, 2007) support the above contention. An anti-inflammatory environment induced by AGP in murine leprosy caused by *Mycobacterium lepraemurium* (Silva Miranda *et al.*, 2006) and in a mouse model of pulmonary tuberculosis (Martínez Cordero *et al.*, 2008) has been shown to favour bacterial growth and disease progression.

High AGP levels and distinct glycosylation patterns have also been shown in another mycobacterial disease, tuberculosis (Fassbender *et al.*, 1995); however, the

biological significance was not explained. These changes also assume significance in the context of drug-binding functions, as AGP binds to thalidomide (Turk *et al.*, 1996) and rifampicin (Johnson & Smith, 2006), two of the drugs commonly used in the treatment of leprosy.

In conclusion, this study showed increased levels of the acute-phase protein AGP and differential expression of its isoforms in ENL patients compared with healthy controls and other stages of the leprosy spectrum, which correlated with the disease and treatment status of the patients. Based on our study, we speculate a possible role of AGP in the ENL stage of leprosy. Increased AGP levels could be a precipitating factor in the development of ENL by inducing increased secretion of pro-inflammatory cytokines such as TNF- α (Boutten *et al.*, 1992). However, the increase in AGP levels could also reflect a physiological feedback inhibition mechanism for inflammation by virtue of increased sLe^x-rich AGP glycoforms, which compete with leukocytes for binding to E-selectin and decrease/inhibit inflammation (De Graaf *et al.*, 1993). However, in ENL, the role of AGP would be more complex as it is known to bind to thalidomide (Turk *et al.*, 1996), and thus the functions of both these immunomodulators would be affected by their interactions. In addition, the presence of thalidomide may have an inhibitory effect on AGP-induced TNF- α production. Thus, interactions of AGP and thalidomide in ENL patients and the concomitant changes in AGP expression and glycoform pattern need further exploration.

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