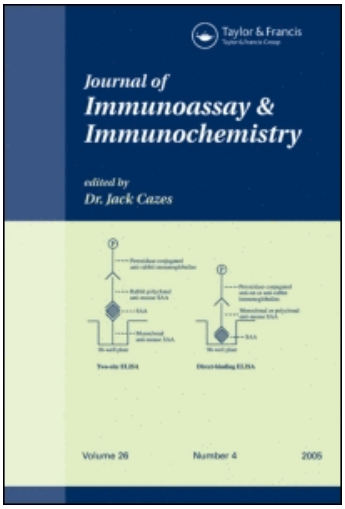

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Attallah, A. M. , Toson, E. A. , Shiha, G. E. , Omran, M. M. , Abdel-Aziz, M. M. and El-Dosoky, I.(2007) 'Evaluation of Serum Procollagen Aminoterminal Propeptide III, Laminin, and Hydroxyproline as Predictors of Severe Fibrosis in Patients with Chronic Hepatitis C', Journal of Immunoassay and Immunochemistry, 28: 3, 199 — 211
To link to this Article: DOI: 10.1080/15321810701454649
URL: http://dx.doi.org/10.1080/15321810701454649

Journal of Immunoassay & Immunochemistry, 28: 199–211, 2007

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810701454649

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Abstract: In an attempt to identify biochemical analytes that could enhance the discrimination between the patients with severe liver fibrosis (F3-F4) and mild fibrosis (F1-F2) based on absolute values of biochemical markers, we measured 12 analytes, including procollagen III aminoterminal propeptide (PIIINP), laminin, proline, hydroxylproline, glycine, AST, ALT, alkaline phosphatase, albumin, total bilirubin, total protein, and prothrombin time in 252 individuals with chronic hepatitis C infection (CHC). PIIINP and laminin were determined by radio-immunoassay; the degraded amino acids were determined using high performance liquid chromatography. Statistical analyses were performed by logistic regression, and receiver operating

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characteristic (ROC) curves. The best linear combination of blood markers was selected by multivariate discriminant analysis (MDA) for construction of the fibrosis discriminant score (FDS). FDS, an index of five markers (PIIINP, laminin, hydroxyproline, prothrombin activity, and AST/ALT) correctly classified 82% of the patients with severe liver fibrosis at a discriminant cut-off score $= -0.5$ (i.e., less than -0.5 indicated severe liver fibrosis and greater than -0.5 indicated mild liver fibrosis with sensitivity (76%) and specificity (89%). This result was reproduced in a validation study with no significant difference. In conclusion, FDS is useful for identifying severe liver fibrosis in patients with CHC.

Keywords: Liver, HCV, Fibrosis, Biomarkers, Score, Procollagen III aminoterminal propeptide

INTRODUCTION

Liver biopsy is currently recommended as the gold standard method of staging fibrosis in patients with chronic hepatitis C.[1]

This procedure, however, is invasive and has potential complications.[2] Non-invasive approaches, developed to assess histological samples, include clinical symptoms, routine laboratory tests, and radiolographic imaging.[3] The serum markers of fibrogenesis include platelet count, albumin level, the ratio of alanine aminotransferase and aspartate aminotransferase levels, and alkaline phosphatase level.[4,5] Another non-invasive approach relies on the measurement of substances that regulate fibrosis or participate in the generation of the liver extracellular matrix. The most applicable include hyaluronic acid,[6] type IV collagen,[7] and procollagen III aminoterminal propeptide (PIIINP).[8] Serum concentrations of procollagen peptides, especially PIIINP, have proved to be a useful, non-invasive measure of the activity of this pathway at a single time point and have been shown to reflect prognosis and response to treatment in patients with a variety of chronic liver diseases.[9] In the liver, total collagen accumulation during the fibrotic or cirrhotic process was measured using a methodology based on the determination of collagen amino acids in liver biopsies and serum samples from patients with liver diseases.[10] Several serum markers have been developed to access fibrogenesis. Efforts have been made to replace liver biopsy with non-invasive markers of liver fibrosis which have been assessed in multiple studies, but questions remain on how their sensitivity and significance are affected by various factors.[11] In the present study, we assessed and compared the diagnostic accuracy of PIIINP, laminin, degradation amino acids, and routine liver function tests as biochemical markers of severe liver fibrosis in chronic HCV patients, using ROC curves. We also developed and evaluated a sensitive and specific fibrosis discriminant score based on these blood markers to predict severe liver fibrosis.

EXPERIMENTAL

Samples

A total of 252 Egyptian individuals (163 males, 89 females; aged 25–58 year) with clinically and laboratory confirmed chronic hepatitis C (CHC) infection were included in the present study. They were recruited from the Mansoura University Hospitals, Mansoura, Egypt that approved the present study. Needle liver biopsy specimens (n = 252) were taken from the patients and examined by a pathologist unaware of the laboratory results. METAVIR score was used to stage the fibrosis (F0 to F4).[12] Blood samples were collected from all patients by vein-puncture within 2 weeks of liver biopsy and a part of the blood was treated immediately with a citrate solution for prothrombin time. Sera were separated from the rest of blood samples and tested for liver function indices. Patients with chronic HCV infection were diagnosed on a positive test for anti-HCV antibody (Ortho HCV EIA; Ortho Diagnostics, Raritan, New Jersey, USA). The HCV infected individuals were pathologically classified into two groups: 192 individuals with severe liver fibrosis (F3-F4) and 60 individuals with mild liver fibrosis (F1-F2). Liver function tests (aspartate aminotransferase [AST] and alanine aminotransferase [ALT], alkaline phosphatase, albumin, total bilirubin, total protein, and prothrombin time) were measured using standard methodologies (bioMérieux SA, Marcy l'Etoile, France). All individuals were negative for other causes of chronic liver disease, including viral hepatitis A and B. None of the patients had other causes of chronic liver injury, a history of habitual alcohol consumption, or hepatocellular carcinoma. The study protocol conforms to the ethical guideline of the 1975 Declaration of the Helsinki.

Measurement of PIIINP and Laminin

Both PIIINP and laminin were measured by radioimmunoassay, employing commercial kits (Behringer AG, Marbyrg, Germany).

Determination of the Concentration of Proline, Hydroxylproline and Glycine using HPLC

The levels of degradation amino acids were determined using a Kontron Pc integrator HPLC system equipped with a spherisorb C_8 colum (205 × 4.6 mm i.d., Kontron, Switzerland) and M742-HPLC detector system with variable wave length capability. Samples were injected in a volume of 10 μL using a Hamilton syringe (Sigma). The amino acids were developed with a linear gradient of acetonitrile in 0.1 M acetate buffer containing 1 ppm EDTA, pH 5.5. The mobile phase used solvent A which was aqueous buffer (0.1 M acetate buffer containing 1 ppm ethylene diamine tetraacetic acid (EDTA),

pH 5.5). Solvent B was an organic phase consisting of acetonitrile-methanol-water (45:40:15). The column was initially equilibrated at 0% at a flow rate of 1.5 mL/minute. The separation was performed using a linear gradient of 6 to 45% during 60 minutes. 20 μL serum samples were used for derivatization with phenyl isothiocyanate (PITC) and dried under vaccum. Samples were re-dried under vaccum, and the procedure was completed according to the method of Stone and Williams.[13]

Statistical Analyses

All statistical analyses were done by a statistical software package "SPSS 12.0 for Microsoft Windows, (SPSS Inc.) and considered statistically significant at a two-sided $P < 0.05$. Numerical data were expressed as mean $\pm$SD. The levels of markers were analyzed by ANOVA, but the Mann-Whitney U-test was used for comparisons between independent groups. Correlation coefficients were calculated to assess the relationship between the histological degree of severe liver fibrosis and the concentrations of serum markers. To assess and compare the diagnostic accuracy of markers for discriminating in chronic HCV patients, those with severe liver fibrosis from those with mild fibrosis, we plotted ROC curves[14] and calculated the areas under the curves (AUC) for comparison. ROC curves were generated by plotting the relationship of the true positivity (sensitivity) and the false positivity (1 - specificity) at various cutoff points of the tests. An AUC of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value.[15] The multivariate discriminant analysis (MDA) was carried out stepwise with use of the minimum Wilks' lambda. The diagnostic sensitivity, specificity, efficiency, and positive predictive (PPV) and negative predictive (NPV) values were calculated.

RESULTS

Patients Characters

In an attempt to identify biochemical analytes that could enhance the discrimination between the patients with severe liver fibrosis (F3–4) and mild fibrosis (F1-F2), we measured 12 analytes PIIINP, laminin proline, hydroxylproline, glycine, AST, ALT, alkaline phosphatase, albumin, total bilirubin, total protein, and prothrombin time. The mean level ($\pm$SD) of PIIINP in U/mL of sever liver fibrosis (F3-F4) was 1.0 ($\pm$0.47) and in mild liver fibrosis (F1-F2) was 0.52 ($\pm$0.12). The mean level of laminin ($\pm$SD) in IU/mL of severe liver fibrosis was 3.52 ($\pm$2.70) vs. 2.32 ($\pm$1.17) in mild liver fibrosis. The mean levels ($\pm$SD) in μmol/100 mL of proline was 22.3 ($\pm$15.1) vs. 25.01 ($\pm$8.72), hydroxyproline was 28.4 ($\pm$15.4) vs. 14.76 ($\pm$3.5), and glycine was 31.6 ($\pm$19.8) vs. 25.76 ($\pm$6.33) for patients and

controls; respectively. The differences between the mean levels of PIIINP, laminin, and hydroxyproline in severe liver fibrosis patients and mild fibrosis were extremely significant ($p < 0.0001$). Proline and glycine levels decreased with the progression of liver fibrosis but did not reach a statistically significant difference in proline ($p > 0.05$) and the difference in glycine was significant at $p < 0.05$. Glycine and proline levels were then excluded from subsequent analyses.

Diagnostic Performance using the ROC Curves

ROC curves were used to determine cut-off values with best efficiency of serum hydroxyproline (20 μmol/100 mL), laminin (2.8 IU/mL), and PIIINP (1 U/mL) and the areas (p value) under the curves were 0.53 (0.547), 0.60 (0.035) and 0.65 (<0.001) for these markers, respectively; see Figure 1. Serum PIIINP was the most efficient index. So, we have been taking serum PIIINP as the basic index to combine with other indices to discriminate between severe liver fibrosis and mild liver fibrosis.

Performance Characteristics of FDS Score

The best linear combination of blood markers was selected by MDA for construction of the fibrosis discriminant score (FDS) equations based on three markers (PIIINP, laminin and hydroxyproline). Therefore, in an attempt to

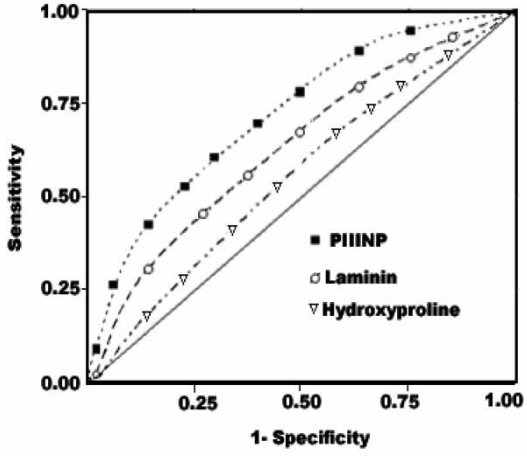

Figure 1. ROC curves of single measurement of PIIINP, laminin and hydroxyl proline for discriminating patients with severe liver fibrosis from patients with mild liver fibrosis. The areas under the curves (p value) are 0.65 (<0.001), 0.60 (0.035), and 0.53 (0.547) for these markers, respectively.

enhance the discrimination power of FDS score, we inserted prothrombin time as the fourth marker (PIIINP, laminin, hydroxyproline, and prothrombin time) then AST/ALT as fifth marker (PIIINP, laminin, hydroxyproline, prothrombin activity, and AST/ALT) for discriminating patients with severe liver fibrosis from patients with mild liver fibrosis. The areas under the ROC curves (p value) of prothrombin activity and AST/ALT were 0.72 (<0.0001) and 0.82 (<0.0001); respectively, Figure 2. The multivariate discriminant analysis (MDA) selected a score based on absolute values of the five biochemical markers; fibrosis discriminant core = [prothrombin activity × 0.06−(2.22 (numerical constant) + PIIINP [U/ml] × 1.15 + Laminin [IU/ml] × 0.163 + Hydroxyproline [μmol/100 ml] × 0.001+ AST/ALT × 2.72) was selected, Table 1.

To evaluate the differential diagnostic power of the discriminant function, we constructed ROC curves for FDS and compared them with ROC curves of the other variables that we had previously found to be significantly different for severe and mild liver fibrosis. FDS, calculated for each patient on the basis of the linear combination of variables selected by MDA, was used to classify cases into one of the two groups (mild and severe liver fibrosis). The correlation values (p value) of hydroxyproline, laminin, PIIINP, prothrombin activity, AST/ALT and FDS with Metavir score fibrosis stage were 0.168 (0.040), 0.203 (0.013), 0.264 (0.002), −0.279 (0.001), 0.491 (<0.0001), 0.632; P < 0.00001; respectively. Figure 3 shows box plots for FDS in patients with mild liver fibrosis (F1-F2) and those who had (F3-F4) severe liver fibrosis. The median, mean and ±SD of FDS in severe liver

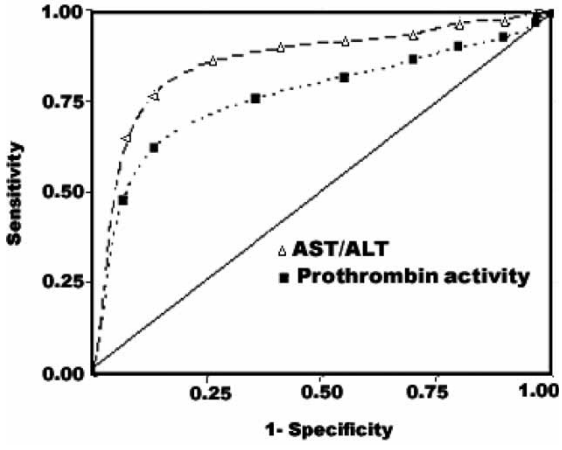

Figure 2. ROC curve of AST/ALT and prothrombin activity for discriminating patients with severe liver fibrosis from those with mild liver fibrosis in chronic viral hepatitis C. The areas under the ROC curves (p value) of these markers were 0.82 (<0.0001) and 0.72 (<0.0001), respectively.

Table 1. Multiple logistic regression model for FDS based on absolute values of five blood markers (porcollagen III, laminin, hydroxylproline, AST/ALT and prothrombin time) for discriminating patients with severe liver fibrosis (F3-F4) from mild liver fibrosis (F1-F2)

Variable	Coefficients	SE[a]	P value	AUC[b]	(95 % CI[c])
Procollagen III	−1.152	0.040	0.001	0.640	0.56–0.72
Laminin	−0.163	0.041	0.035	0.589	0.51–0.67
Hydroxyproline	−0.001	0.042	0.547	0.525	0. 44–0.61
Prothrombin activity	0.063	0.038	<0.0001	0.72	0.64–0.79
AST/ALT	−2.721	0.031	<0.0001	0.82	0.750–0.871

[a]SE = Standard error.
[b]AUC = Area under (ROC) curve.
[c]CI = Confidence interval.

fibrosis patients were −1.91, −1.89 and ±2.12 and in mild liver fibrosis patients were 1.15, 1.14, and ±1.46; respectively. The figures were extremely significant ($P < 0.0001$) when comparing value of FDS in severe liver fibrosis patients and in mild liver fibrosis. The areas under the curves were 0.73, 0.75, 0.81 ($P < 0.0001$) for three, four and five markers; respectively, Figure 4. The fibrosis discriminant score (FDS) correctly classified 82% of the patients with severe liver fibrosis at a discriminant cut-off

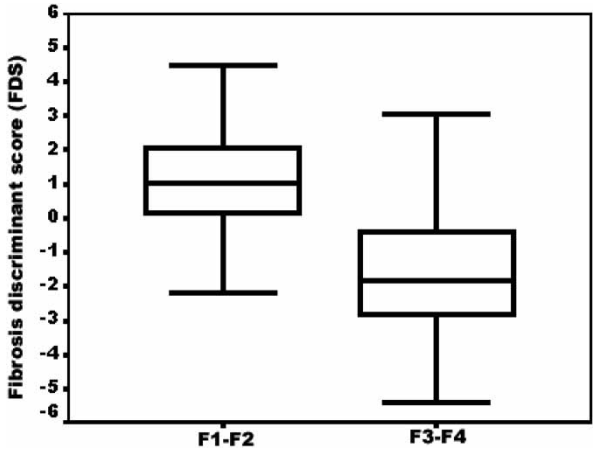

Figure 3. Box plots for fibrosis discriminant score (FDS) in patients with mild liver fibrosis (F1-F2) and those who had (F3 to F4) severe liver fibrosis. The box represents the interquartile range, the whiskers indicate the highest and lowest values, and the line across the box indicates the median value. Overall significance of differences among the two groups was determined by ANOVA for FDS $P < 0.0001$.

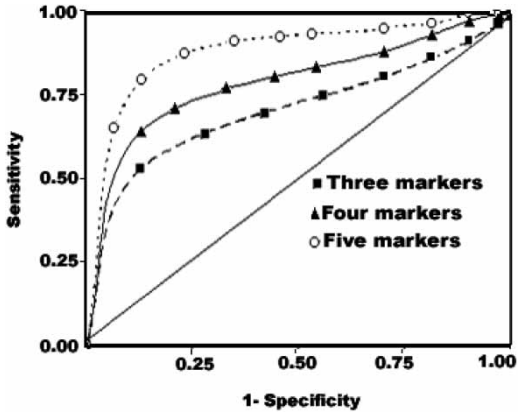

Figure 4. ROC curves for three combined markers (PIIINP, laminin and hydroxyproline) and four markers (PIIINP, laminin, hydroxyproline and prothrombin time) and five markers (PIIINP, laminin, hydroxyproline, prothrombin activity and AST/ALT) for discriminating patients with severe liver fibrosis from patients with mild fibrosis. The areas under the curves are 0.73, 0.75, 0.81 ($P < 0.0001$) for three, four, and five markers, respectively.

score $= -0.5$ (i.e. less than -0.5 indicated severe liver fibrosis [F3-F4] and greater than -0.5 indicated mild liver fibrosis [F1-F2) with sensitivity (76%) and specificity (89%). The positive and negative predictive values were 90% and 74%; respectively, Table 2.

Validation Study

In a second part of the study, we evaluated whether the predictive criteria identified in the first part were able to reproduce their predictive ability in a subsequent different, but related group of patients. A total of 150 patients (92 males, 58 females; aged 25–58 year) were included. Clinical and pathological investigation, as well as inclusion and exclusion criteria for the study, and classification adopted in this second group of patients were the same as those used in patients in the first part. In practice, the FDS applied to the second cohort of patients revealed that 89% of patients with mild liver fibrosis and 75% of patients with severe liver fibrosis were correctly classified. The positive and negative predictive values were 94% and 62%; respectively.

DISCUSSION

Hepatitis C virus infection is now becoming a common health problem in both developed and developing countries, especially in Egypt.[16] The main

Table 2. Performance characteristics of different discriminant scores derived from MDA function based on absolute values of five blood markers (porcollagen III, laminin, hydroxylproline, AST/ALT and prothrombin time) for discriminating patients with severe liver fibrosis (F3-F4) from mild fibrosis (F1-F2)

MDA[a] cut-off score	Performance characteristics				
	Sensitivity	Specificity	PPV[c]	NPV[d]	Efficiency
2	98	27	63	92	67
1.5	97	39	67	92	72
1	91	54	71	82	74
0.5	87	69	78	81	79
0	81	81	84	77	81
[b]−0.5	76	89	90	74	82
−1.0	70	93	93	71	80
−1.5	59	95	94	65	75
−2.0	48	96	94	60	69

[a]MDA = Multivariate discriminant analysis.

[b]Selected as a discriminant score at −0.5 (i.e. less than −0.5 indicated liver severe liver fibrosis [F3-F4] and greater than −0.5 indicated mild liver fibrosis [F1-F2]).

[c]PPV = Positive predictive value.

[d]NPV = Negative predictive value.

treatment goal in patients with chronic HCV infection is the prevention of progressive hepatic fibrosis by early diagnosis.[17] There is a clinical need for noninvasive measurement of liver fibrosis both to diagnose significant liver fibrosis and to monitor the effects of therapy on fibrogenesis and fibrolysis.[18,19] The measurement of non-invasive markers of collagen synthesis and degradation may be useful in monitoring the fibrosis process.[20] The most often used markers of hepatic fibrogenesis are type III procollagen laminin and type IV collagen.[21] The PIIINP, laminin and hydroxyproline are measurable in serum and are now considered useful serum markers of fibrogenesis and inflammation in chronic liver diseases.[22–24] However, very few studies, thus far, have focused on assessing the diagnostic value of these markers in detecting severe fibrosis in chronically diseased liver. Therefore, we aimed to compare its diagnostic values in detecting of severe liver fibrosis in a homogeneous group of 192 patients suffering from CHC. The difference between the means of PIIINP, laminin and hydroxyproline in patients with mild and severe liver fibrosis were extremely significant ($p < 0.0001$). This could be explained as HCV induces an increased expression of factors that differentially modulate hepatic stellate cell expression of key genes involved in liver fibrosis in a clearly profibrogenic way, up-regulating procollagen alpha1 (I) and procollagen alpha1 (III) and down-regulating fibrolytic matrix

metalloproteinases.[25] Laminin concentration was increased in early stages of chronic liver disease, possibly as a marker of regeneration; the highest concentrations were in active cirrhosis and chronic active hepatitis.[26,27] The increase in hydroxyproline might indicate that an increased proportion of newly synthesized collagen in the fibrosis.[10] Here, levels of serum PIIINP, laminin, hydroxyproline and liver function tests were correlated. The observed correlation seems to confirm the metabolism of collagens.[28] ROC analysis has been used to compare the ability of the assays to detect advanced liver injury.[29] Based on the ROC curve analysis for discriminating patients with severe liver fibrosis, areas under the curves of PIIINP were 0.69[30] and 0.80.[25] In our study, ROC curves showed that PIIINP and laminin were more efficient than hydroxyproline in identifying severe liver fibrosis with area under curve were 0.65, 0.60 and 0.53; respectively. AST/ALT ratio and prothrombin activity were included in the MDA score because they have shown good diagnostic accuracy in patients with chronic viral liver disease.[4,31] MDA based on serum biochemical indices was previously used as independent predictors of fibrosis.[32] The diagnostic value of the five laboratory markers was assessed using logistic regression analysis, sensitivity, specificity and predictive values, and a score was constructed combining the most significant factors identified. In our study, the fibrosis discriminant score, an index of five markers (PIIINP, laminin, hydroxyproline, prothrombin activity and AST/ALT) with areas under the curves was 0.81, correctly classified 82% of the patients with severe liver fibrosis at a discriminant cut-off score $= -0.5$ (i.e., less than -0.5 indicated severe liver fibrosis and greater than -0.5 indicated mild liver fibrosis with sensitivity (76%) and specificity (89%). The positive and negative predictive values were 90% and 74%, respectively. Hence, this FDS enabled us to accurately predict the presence of severe liver fibrosis in patients infected with HCV. The area under the receiver operating characteristic (ROC) curves of our combinations was similar to other report.[33] However, negative predictive value is lower than the model developed by Forns et al.[34] and Attallah et al.[35] We demonstrated that the use of the FDS maintained its accuracy in the non-invasive diagnosis of fibrosis. Indeed, 75% of the patients who had FDS < -0.5 were patients with severe liver fibrosis while 89% of patients with mild liver fibrosis with FDS > -0.5. The positive and negative predictive values were 94% and 62%, respectively. The positive predictive value for validated FDS was higher, but the negative predictive value was lower than other report.[36] The false negative and false positive results of FDS can be explained in the light of studies described in reference [37]. In conclusion, FDS reflects some aspects of extracellular matrix synthesis and degradation. FDS, as noninvasive diagnosis technique, can be applied to patients who either have contraindications or refuse liver biopsy for the management of their HCV infection.

ACKNOWLEDGMENT

The authors would like to thank Dr. Kadry S. Gad at Faculty of Medicine, Mansoura University for his kind assistance.

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Received December 20, 2006
Accepted January 27, 2007
Manuscript 3224