Longitudinal Measurement of Methotrexate Liver Concentrations Does Not Correlate with Liver Damage, Clinical Efficacy, or Toxicity During a 3.5 Year Double Blind Study in Rheumatoid Arthritis

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ABSTRACT. ***Objectives.*** In patients with rheumatoid arthritis (RA), we examined whether methotrexate (MTX) and MTX polyglutamate accumulation in the liver correlated with clinical efficacy or clinical/laboratory toxicity. We also began preliminary examination of a new histologic index of liver histology (the Iowa Score) relative to the Roenigk grading system.

Methods. Forty patients with RA participated in a prospective, double blind, 3.5 year study of MTX treatment. Liver biopsies, liver MTX and MTX polyglutamate concentrations, laboratory tests, evaluation of disease activity, and evaluation of adverse events were done prospectively at baseline and at 1, 2, and 3.5 years. Biopsies were examined using the Roenigk grading system and an additional histological scoring system. Radiochemical ligand binding assays and HPLC methods were used to measure MTX and MTX polyglutamates. Statistical analysis included ANOVA, linear regression, and logistic regression modeling.

Results. No significant changes in the mean values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, albumin, or hemoglobin occurred. A significant percentage of patients had at least one abnormal alkaline phosphatase, AST, or ALT (25 to 52%), although most abnormalities were small and transient. Histological abnormalities did not progress using either the Roenigk or the Iowa score. The last abnormal AST, the number of abnormal AST and ALT, and female sex correlated with histological liver abnormalities ($r^2 = 0.41$) using a new preliminary histologic scoring system (the Iowa Score). Amount of alcohol use correlated with fatty change, and the MTX dose at biopsy was associated with liver histological abnormalities ($p = 0.03$ and 0.049, respectively). Total liver MTX concentrations were stable from Year 1 to Year 3.5 and the percentage of higher order polyglutamates was relatively high (38 to 56%) relative to monoglutamates. No correlation of these concentrations with clinical response or toxicity, histology, or liver function tests could be documented.

Conclusion. This analysis describes the accumulation and stabilization of MTX concentrations in the liver and examined correlations between MTX liver concentrations, patient demographics, liver histology, concomitant medications, and disease activity. No such correlations were found, decreasing the likelihood that MTX concentrations in serum would be useful measures to predict significant hepatotoxicity. (J Rheumatol 2002;29:2092–8)

Key Indexing Terms:
LIVER TOXICITY METHOTREXATE
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Methotrexate (MTX) is the most commonly used disease modifying antirheumatic drug (DMARD) in the treatment of rheumatoid arthritis (RA) today. Its longterm use requires longterm monitoring, and the hepatic safety of longterm MTX therapy continues to be of some concern[1–4].

Early recommendations for periodic liver biopsies were based on the experience in patients with psoriasis who were given MTX[5,6]. Guidelines formulated by the American College of Rheumatology (ACR) suggested regular monitoring of serum aspartate aminotransferase (AST) and albumin at 4 to 8 week intervals. They also reported that liver enzyme values were likely to be abnormal in patients

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with RA treated with MTX who developed clinically significant liver disease[7].

Kremer, *et al*, in a cross sectional study, demonstrated that MTX polyglutamates increased in the liver of patients with RA treated for more than one year with MTX[8]. It was felt that the polyglutamate forms of MTX might have greater cytotoxic effects because they are retained for longer than the parent compound and they accumulate in the liver of patients with RA taking MTX[8-12]. This study prospectively examined liver biopsies longitudinally in a group of patients treated with MTX. It included some baseline biopsies, and related liver MTX concentrations to liver function tests, histological abnormalities, patient characteristics, disease activity, and concomitant medications.

MATERIALS AND METHODS

Study design. This manuscript is an analysis of the data from a parallel, randomized, double blind study of oral MTX use over 3.5 years. After signing fully informed voluntary consent, 52 patients satisfying ACR criteria for definite or classical RA were admitted to the University of Iowa Clinical Research Center for 12 days, where baseline studies and pretreatment liver biopsies were done[11,12]. Background nonsteroidal antiiflammatory drug (NSAID) and stable prednisone therapy were allowed, although the NSAID was stopped for 5 serum half-lives before the pretreatment liver biopsies. The initial 18 weeks of this study were a double blind, placebo controlled dose ranging study and the results have been published[3]. All patients had previously failed gold, D-penicillamine, antimalarials, and/or azathioprine. Beyond 18 weeks, placebo patients were blindly rerandomized to either 5 or 10 mg/m^2 oral MTX taken weekly. Patients taking MTX continued that drug, in double blind fashion. Beyond the initial 18 weeks, for the ensuing 3.5 years, weekly doses of MTX could be varied according to clinical need or toxicity, although both the patient and the physician remained blinded to the MTX dose. When the dosing regimen was changed, the pharmacy was asked to "change the dose by 2.5 mg." All patients were ingesting 14 identical appearing tablets weekly; some were placebo and some were MTX. The pharmacy personnel changed the dose within the 14 tablets, thus maintaining the blind for both patient and investigator. The maximum dose of MTX was 35 mg orally per week.

Baseline tests included liver biopsy, complete blood count, erythrocyte sedimentation rate (ESR), blood liver function tests and renal function tests, plus radiographs of the chest, hands and feet. Followup liver function and complete blood count laboratory tests were done monthly for one year and then every 2 months. If the AST or alanine aminotransferase (ALT) rose to > 3 times normal, test drug was discontinued until the abnormality returned to within normal limits. The MTX could, optionally, be restarted at a lower (although still blinded) dose thereafter. Permanent discontinuation of drug was decided on a clinical basis. Liver biopsies were done after one year, 2 years, and at 3.5 years. No patient was discontinued from the study because she/he developed any specific liver biopsy abnormalities.

Ritchie tender and swollen joint counts, duration of morning stiffness, time to walk 50 feet, physician's global assessment of disease activity, patient's global assessment of disease activity, time to onset of fatigue, and patient pain assessment were all done on 100 mm horizontal visual analog scales (VAS). The patient's ability to complete 18 activities of daily living, each on a scale of 1 to 10, was also assessed.

MTX assays. Four assays were used to measure MTX.

The radio ligand assays used are modifications of the radiochemical ligand binding assay developed by Kamen, *et al*[13].

MTX (Sigma Chemical Co., St. Louis, MO, USA) and H^3-MTX Perkin-Elmer Life Sciences, Boston, MA, USA; specific activity > 35 Ci/mmol) standards were prepared on 0.1 mM KH_2PO_4, pH 7.0 (99% purity, verified by high pressure liquid chromatography, HPLC).

To measure concentrations > 20 pmol/ml in serum, dihydrofolate reductase (DHFR) (Sigma) from chicken liver was used and adjusted so that 0.1 ml DHFR bound 70–80% of 1 pmol ^{3}H-MTX, in the presence of 50 μM NADPH. A 0.1 ml sample was mixed with 0.1 ml of 0.1 mM KH_2PO_4, pH 7.0, 0.1 ml 10 pmol/ml^3 DHFR with NADPH. This was incubated in an ice bath for 5 min. The reaction was stopped with 200 μl dextran coated charcoal, centrifuged at 320 g for 15 min, decanted into 7–10 ml Budget-Solve (Research Products Inc., Mount Prospect, IL, USA), and counted on a Packard Tri-Carb 4530 scintillation counter between 2 and 19 KeV. Results were calculated versus a simultaneously run standard curve. Cross reactivity of this assay with 7-OH-MTX was 0.38% and with folates or folinic acid was < 0.006%. Coefficients of variation (CV) are 5.0% at 25 pmol/ml, 3.4% at 500 pmol/ml, and 4.8% at 1000 pmol/ml.

For MTX concentrations between 2 and 20 pmol/ml, a sequential reaction was used: 0.1 ml DHFR with NADPH was mixed with the 0.1 ml sample and incubated in an ice bath for 20 min; 0.1 ml ^{3}H-MTX was added and mixed with the above. The mixture was again incubated in an ice bath for 10 min. The reaction was terminated and assayed thereafter as for the competitive assay. CV were 4.4% at 2 pmol/ml, 1.2% at 8 pmol/ml, and 7.2% at 20 pmol/ml, despite the above purification procedures. In those sera, the MTX values ≤ 1 pmol/ml were discarded.

For measurement of liver MTX and MTX polyglutamates (MTX-glu), samples of liver were rinsed in ice cold saline, pat dried, weighed in 1.5 ml polypropylene screw-top microfuge tubes, and homogenized in an ice bath. Thirty volumes of extraction buffer were added. The extraction buffer was ß-mercaptoethanol, 10 mM EDTA, and 50 mM Tris buffer (pH 8.3). The sample was rehomogenized on ice and remixed. It was then freeze thawed at –80˚C for 2 min, boiled for 10 min in the screw-cap vial, cooled on ice for 10 min, and centrifuged at 11,300 g for 2 min. Fifty to 100 ml of supernatant were injected directly onto the chromatograph, and analytes were eluted at a flow rate of 1 ml/min using 100 mM KH_2PO_4, pH 7.0, mobile phase. The HPLC apparatus consisted of a Beckman 110 pump, variable-volume injector (Altex 210), an Aminco Flouro-Monitor, and a Pharmacia Frac 100 collector. Thirty 0.5 ml units of eluate were assayed using the above radio ligand assay. Additionally, one 0.05 ml unit of original supernatant was assayed by the radio ligand assay for total MTX.

The sample chromatogram is compared to a standard chromatogram of mono-, di-, tri-, and ≥ 4 MTX polyglutamates, which were expressed as percentages of total MTX in the sample. Later serum assays also utilized a modification of the HPLC method of Stout, *et al*[14]. To an 0.5 ml serum aliquot, 0.1 ml ^{3}H-MTX internal standard was added. Then 1.0 ml acetonitrile was dripped in, vortexed, and transferred to a 1.5 ml polypropylene microfuge tube. This was centrifuged at 11,300 g for 2 min. The supernatant was decanted into a second 1.5 ml microfuge tube and centrifuged at 5˚C, 50 g for 5 min. It was then placed in an ice bath for 30 min, to allow for separation of the extract. The upper, separated acetonitrile layer was aspirated and discarded, and the aqueous portion was vacuum dried.

The residue was reconstituted with 0.1 ml mobile phase (see below), solvated, microcentrifuged at 10,000 g at room temperature for 1 min, and transferred to hexamethyldesilicized glass autosampler vials. The HPLC system was a Beckman 334 Gradient LC with a Beckman 504 autosampler, a Kratos Spectroflow 757 UV detector (at 313 nm), and Shimadzu C-R1B Integrator. It was fitted with a 15 cm, 4.6 mm ID, size-exclusion 300 × 6.4 mm column [self-packed using 3 μm ODS Hypersil (Phenomenox Inc.)] at 8000 psi, and a slurry column packer (Thermo Electron Corp., Waltham, MA, USA). One milliliter samples were automatically injected at 20 min intervals. The mobile phase was 0.1 M KH_2PO_4– 10 mM Tris phosphate (pH 7.0), methanol, and acetonitrile in volume ratios of 1000:33:66, respectively. CV were 4.9% at 50 pmol/ml, 3.4% at 250 pmol/ml, and 3.2% at 750 pmol/ml.

Stability of MTX and polyglutamate levels was documented by repeated analyses of 2 biopsies over a 2 year period. Concentrations of MTX and polyglutamates varied less than the CV for up to 2 years. Repeated sampling for stability beyond 2 years was not done.

Description of liver histology. Biopsy specimens were fixed in Bouen fixative, and paraffin embedded sections were stained with hematoxylin and eosin, Masson's trichrome, and PAS after diastase digestion.

Liver biopsy histology was scored according to 2 systems: (1) the Roenigk classification included the following: grade I was normal, although mild fatty infiltration, nuclear variability, and portal inflammation were allowed; Grade II required moderate to severe fatty infiltration, nuclear variability, portal tract inflammation and necrosis; grade IIIA required mild fibrosis, slight enlargement of portal tracts, and some formation of fibrotic septae; grade IIIB required moderate to severe fibrosis with all other changes of grade IIIA; grade IV described cirrhosis[15,16]. (2) The Iowa classification is an attempt to increase the sensitivity of the Roenigk histological scoring method. It examined anisocytosis, fatty change, portal inflammation, hepatocellular necrosis and fibrosis, and septa formation, each on a zero to 3+ scale. It multiplies the anisocytosis score and fatty change score by 1, portal inflammation and necrosis scores are each multiplied by 2, and the septa formation score is multiplied by 3 (maximum equals 9). The Iowa Score has face validity, is reproducible, and is quite easy to use. In this report, its construct validity was examined against the Roenigk classification over a limited range of changes in liver histology.

Statistical analysis. Descriptive analysis was done at baseline for all demographic measures, RA disease activity measures, liver function tests, and liver histologic and MTX concentration variables.

Analysis of variance (ANOVA) was used to examine MTX and polyglutamate concentrations in the liver biopsy over time. Dunnett's test was used if the F values from the ANOVA were significant.

Linear regression was initially used to examine relationships between MTX concentrations in the biopsy and the histological scoring systems, the MTX dose at biopsy, the mean MTX dose during each period of time prior to a biopsy (1 to 1.5 years between biopsies), and the total amount of the MTX used over time. Simple linear regression was also used to explore the relationship between liver function tests and histological scoring systems[15,16].

The selected variables chosen from the simple linear regression were then subjected to multiple linear regression for examination of the relationship between liver function tests (ALT, AST) and histology. The histological variables tested included the Roenigk and Iowa classification systems, nuclear variability, portal inflammation, hepatocellular necrosis, fibrosis, septa formation, and the presence or absence of Ito cells. Multiple linear regression was also used to examine the relationship between the concentration of MTX and its polyglutamates in the liver biopsies and the following variables: the biopsy year, patient age, disease duration, sex, weekly MTX dose at biopsy, mean MTX dose over the year (or 1.5 years) previous to the biopsy, the total amount of MTX used, prednisone usage, joint swelling counts, and ESR.

Finally, logistic regression was used to examine the relationship between the details of liver histology (described above) in terms of whether those changes became worse or stayed the same/better (dependent variables) against the following independent variables: mean AST, ALT or alkaline phosphatase, number of abnormal AST and ALT, alcohol use (yes or no), NSAID use (yes or no), MTX concentration in the biopsy, diglutamic MTX or > 2 Glu MTX concentrations in the biopsy, use of prednisone (yes or no), prednisone dose (mg/day), weight (kg), ESR (mm/h), joint swelling count, joint tenderness count, patient global estimation of disease activity on 100 mm VAS, and activities of daily living (0–180). Unfortunately, folic acid was not included as an independent variable in the analysis.

RESULTS

Although 52 patients consented to the study, only 46 were evaluated for efficacy because 6 patients dropped out before receiving any MTX (2 patients had traumatic baseline liver biopsies and discontinued the study, and 4 decided not to proceed). An additional 6 patients dropped out before their 1 year biopsy (they either dropped out during the placebo controlled portion or they developed other toxicities precluding study continuation). Thus 40 patients are evaluated in this dataset. Unfortunately, the validation of the assay and a laboratory accident resulted in the loss of all but 10 baseline liver biopsies; larger numbers of later biopsies were available to be assayed and examined (see tables).

There were 27 women and 13 men, with a mean disease duration of 12.6 ± 9.0 (mean ± standard deviation) years, age 54.6 ± 8.8 years, weight 72.4 ± 14.5 kg; 87.5% were rheumatoid factor positive. While 16 patients (40%) drank alcohol, none drank more than 2 ounces weekly. Only one of 40 patients had diabetes, controlled by oral agents. Sixty-five percent were using prednisone, with a mean dose of 4.0 ± 4.1 mg/day. All patients had failed gold therapy, 80% had failed D-penicillamine, 60% had failed azathioprine, and 55% had used antimalarials. At the time of this study, combination DMARD therapy was uncommon and no patient was using combination DMARD. Eighteen patients were taking stable NSAID.

Table 1 outlines the patients' baseline disease activity and the changes in disease activity during the 3.5 years of followup. As expected, the number of patients followed decreased during the study. At 3.5 years, 77.5% were still in the study. Also as expected, those patients remaining experienced improvement in their disease activity. No specific statistical analysis was done on disease activity, as this was not a primary focus of this study. Nevertheless, it appears clear that those patients remaining in the study improved relative to baseline.

Table 2 shows selected laboratory data during the 3.5 year followup. No significant changes in the mean values of AST, ALT, alkaline phosphatase, albumin, or hemoglobin occurred. There were also no significant mean changes in serum creatinine, white blood cell count, or platelet count during the followup period (data not shown).

Table 3 shows the percentage abnormal values for liver enzyme determinations during the study. There was a surprisingly high percentage of abnormal liver function tests during the study. These abnormalities were frequently single, small increases above normal or irregular increases in liver function tests that returned to normal at the next evaluation without change in therapy, and thus did not result in study discontinuation. There were trends for the AST and ALT to be reflected in the Roenigk score ($p = 0.07$ for each), but they never reached statistical significance. No other correlation was found between the Roenigk score and laboratory values.

Using multiple linear regression, the value of the last abnormal AST was reflected in the Iowa score ($p = 0.007$). The number of abnormal AST findings and female sex significantly affected the Iowa score ($p = 0.0001$ for AST and $p = 0.026$ for sex). The coefficient of determination for this model was 0.41, indicating that 41% of the variability of the Iowa score was predicted by AST and sex. ALT was not an

Table 1. Clinical and demographic features of patients (mean ± SD).

	Baseline	N	1 Year	N	2 Years	N	3.5 Years	N
Ritchie joint tenderness count (maximum = 93)	60.6 (28.5)	40	21.9 (17.5)	39	15.1 (14.8)	38	9.35 (9.0)	31
Ritchie joint swelling count (maximum = 78)	37.5 (14.4)	40	17.6 (11)	39	12.8 (10.5)	38	6.56 (6.3)	31
ESR, mm/h	62.9 (35.6)	40	42.9 (26.3)	39	32.3 (26.0)	38	27.1 (20.6)	31
Patient global VAS, (maximum = 100 mm)	63.0 (15.4)	40	36.07 (15.6)	39	34.1 (19.1)	38	35.8 (17.3)	31
Patient pain VAS (maximum = 100 mm)	65.2 (20.4)	40	35.1 (16.9)	39	31.6 (18.7)	38	34.8 (18.9)	31
Physician global VAS (maximum = 100 mm)	63.2 (14.3)	40	30.2 (14.07)	39	27.3 (15.9)	38	30.0 (16.0)	31
Activities of daily living (maximum = 180)	109 (25.0)	40	64.9 (32.6)	39	59.7 (34.2)	38	60.3 (34.1)	31

Table 2. Patient laboratory data (mean ± SD).

	Baseline	N	1 year	N	2 years	N	3.5 years	N
Hemoglobin, g %	12.6 (1.6)	40	13.1 (1.9)	39	13.59 (1.7)	38	14.09 (1.6)	32
Alkaline phosphatase, < 105 IU/ml	96.5 (28)	39	98.1 (31.7)	38	101.4 (31.98)	38	96.09 (33.37)	30
AST, < 40 IU/ml	23.6 (10.4)	39	35.1 (25.01)	38	35.2 (44.05)	38	26.8 (21.6)	29
ALT, < 35 IU/ml	11.9 (10.4)	39	33.6 (49.9)	38	32.2 (61.88)	37	25.7 (27)	29
Albumin, g %	3.8 (0.3)	39	3.9 (0.88)	37	4.09 (0.277)	37	4.07 (0.39)	29

Table 3. Fraction abnormal (Abnl) liver function tests (> upper limit of normal).

Year	Alkaline Phosphatase		AST		ALT	
	Fraction	NAbnl/ NTested	Fraction	NAbnl/NTested	Fraction	NAbnl/NTested
1	0.27	125/458	0.27	123/456	0.33	146/442
2	0.51	183/359	0.26	94/362	0.46	154/338
3.5	0.52	103/198	0.25	29/112	0.48	92.192

independent contributor to the Iowa score (while AST was).

Table 4 displays the results of liver biopsies, scored according to the Roenigk classification. It can be seen that there was no progression in the liver histology over time in these patients. No mean progression of abnormal histology is seen, although there appeared to be some slight increase in the fibrosis over time — grade III changes were found, but no grade IV changes (cirrhosis) were seen[15,16].

Table 5 displays the same results according to the Iowa classification. Again, there was no progression in the liver histology over time in these patients. No consistent relationship was found using multiple linear regression between the Iowa or Roenigk score and any histological variable, including fibrosis and septa formation. When logistic regression analysis was done on the Roenigk score as well as the individual histologic characteristics of the Iowa score or the overall Iowa score, the amount of alcohol use was found to significantly determine worsening of fatty change in the liver ($p = 0.03$) and the MTX dose at biopsy appeared to affect the Iowa score ($p = 0.049$). However, no relationship was found among any of the above and the mean AST, ALT, alkaline phosphatase, number of abnormal AST or ALT, MTX or polyglutamate concentrations in the liver, NSAID use, prednisone use, prednisone dose, weight, ESR, joint swelling count, joint tenderness count, patient global assessment of disease activity, or activities of daily living.

Table 6 shows the concentrations of MTX and its polyglutamates in 119 liver biopsies of patients with RA. Although the total MTX in the liver appeared to remain stable from year 1 through year 3.5, the percentages of

Table 4. Liver histology according to the Roenigk classification.

	N	Mean Roenigk Score	Range	No. with Grade IIIA Fibrotic Changes (no IIIB Found)	No. with Grade IV Fibrotic Changes
Baseline	10	1.4	1–2	0	0
1 year	39	1.26	1–2	2	0
2 year	38	1.36	1–3	3	0
3.5 year	32	1.38	0–3	4	0

Table 5. Liver histology, according to the Iowa classification (see text for details). Data are mean (± SD).

	Baseline, N = 10	1 Year, N = 39	2 Years, N = 38	3.5 Years, N = 32
Anisocytosis	1.15 (0.66)	1.0 (0.5)	1.4 (0.7)	1.4 (0.9)
Glycogen storage	0.4 (0.69)	0.2 (0.4)	0.5 (0.8)	0.6 (0.9)
Fatty change	0.25 (0.42)	0.5 (0.6)	0.6 (0.5)	0.5 (0.6)
Mitoses	0	0.8 (0.4)	0	0.03 (0.2)
Portal inflammation	0.65 (0.9)	0.54 (0.8)	0.3 (0.6)	0.5 (0.8)
Hepatic cell necrosis	0.1 (0.31)	0.01 (0.08)	0.03 (0.1)	0.03 (0.2)
Septa	0	0.026 (0.11)	0	0

higher order polyglutamates were relatively high for the first 2 years (38–56%) compared to the MTX to which only one glutamate was added (17–21%) ($p \leq 0.05$, ANOVA). At year 3.5 these concentrations equalized. As noted above, no relationship was found between MTX and polyglutamate concentrations in the liver and any specific histological change, disease activity measure, liver function tests, or concomitant medication use. The use of folic acid also did not affect total MTX concentrations in the liver ($p = 0.58$, linear regression).

Table 7 outlines MTX dosing regimens in patients. The dosing regimen remained stable for at least one month prior to each biopsy and never changed by more than one tablet for the 2 months prior to any biopsy. Dosing appeared to be stable throughout the followup period.

DISCUSSION

This study is the first to examine longitudinal liver MTX and metabolite concentrations and to attempt to correlate those with liver histology, liver function test abnormalities, measures of disease activity, and concomitant medications used to treat RA. Patients were followed prospectively and given MTX in a blinded manner over 3.5 years, with repeated liver biopsies. While relatively frequent transient liver test abnormalities were found (Table 3), no mean changes in liver function tests were observed. Further, no mean changes in liver histology were seen and no progression to cirrhosis was found. Finally, while some accumulation of higher order polyglutamates of MTX was seen over the 3.5 years of the study, no correlation of these concentrations with histology or liver function tests could be documented.

Whiting-O'Keefe, *et al* performed a metaanalysis of 15 studies regarding the progression of histopathologic changes in patients receiving MTX[17]. The studies included 636 patients treated with MTX who had a combination of diseases, including psoriasis, psoriatic arthritis, RA, and other arthropathies. They reported that the progression of liver disease was associated with the cumulative dose of MTX ($p = 0.01$). While the development of advanced histologic changes was not associated with the cumulative dose of MTX ($p = 0.08$), patients who were heavy users of alcohol (at least 100 g alcohol/week) were more likely to have advanced changes on liver biopsies ($p = 0.0003$). The other major risk factor for severe liver disease (Roenigk Grade IIIB or IV) was the presence of diabetes. Wilkins, *et al* reported there were mild histological abnormalities in 29% of the liver biopsies in 52 patients, while 60% of those with Roenigk grade IV had hypoalbuminemia[18]. Initiation of MTX therapy and subsequent drug accumulation sometimes led to fibrosis, a process that some[19,20] but not all[21,22] investigators have found to be accelerated by alcohol consumption and excess body weight. No significant hepatocellular or other damage was found in liver biopsies in 4 other studies, but all documented that there were risk factors for development of severe liver disease in patients with RA, such as diabetes and alcohol consumption[1,18,19,23].

Our study did not address most of these risk factors so no comment can be made regarding diabetes or alcohol use and liver histology. Only one of the 40 patients was diabetic, and alcohol use was limited (at least by history) to < 2 ounces/week among the 40% of patients who drank any alcohol at all. The mean weight of our patients was 72.4 kg, obviating a major influence of obesity (although the range of weight was 42 to 105 kg).

Liver failure and cirrhosis have been reported among

Table 6. MTX and its polyglutamates in the liver (pmol/mg liver). Data are mean (± SD)

	Year 1			Year 2			Year 3.5		
	N	Pmol/mg	% of Total	N	Pmol/mg	% of Total	N	Pmol/mg	% of Total
Total MTX	35	1.69 (1.07)	—	34	1.71 (1.11)	—	31	1.64 (1.15)	—
Monoglutamate	28	0.30 (0.40)	17 (15)	21	0.49 (0.43)	21 (20)	16	0.53 (0.48)	33 (20)
> 1 glutamate	28	0.95 (0.86)	56 (26)	21	0.85 (1.15)	38 (34)	16	0.47 (0.36)	31 (16)

Table 7. MTX dosing. Mean (± SD).

	No. of Biopsies	Mean MTX Dose Prior to Biopsy, mg	Mean Total MTX at Time of Biopsy, mg
Baseline	10	0	—
Year 1	39	16.7 (6.7)	767 (288)
Year 2	38	15.8 (7.9)	1427 (606)
Year 3.5	32	15.9 (5.6)	2987 (686)

patients with RA[24-26] and cases have been reported among RA patients who received MTX therapy[27]. On the other hand, patients demonstrate a variety of mild mesenchymal and parenchymatous abnormalities of the liver in the absence of MTX therapy[27]. Serial serum AST elevation and decreases in serum albumin may be associated with fibrosis[28]. In the study of Weinblatt, *et al*, liver biopsies were performed in 17 patients at 24 months, in 15 patients at 48 months, and in 10 patients at 72 months[1], although liver MTX/polyglutamate concentrations were not obtained. At the time of the first biopsy, after 24 months and with a mean cumulative MTX dose of 1.082 ± 1.049 g, there was no evidence of fibrosis or cirrhosis. In the 15 patients who underwent a second biopsy after 48 months of therapy, the cumulative MTX dose was 2.006 g. Results in 13 biopsies were graded as class I, in one biopsy as class II, and in one biopsy as class IIIA (mild fibrosis). At the time of the third liver biopsies in 10 patients, the cumulative dose was 3.095 ± 0.315 g. Seven specimens were graded as class I, 2 as class II, and one as class IIIA. There was no case of cirrhosis or moderate fibrosis in this cohort. As in the above studies, no patient developed cirrhosis in our cohort.

Our study extends previous work because it looks at longitudinal measures of histology and liver MTX concentrations and seeks to correlate these to laboratory and clinical variables. We found no effects of numerous variables on liver histology, including MTX concentration in the liver, use of medications such as NSAID or prednisone, and measures of disease activity. The use of alcohol did influence fatty change in the biopsy, as expected. There was no correlation of fatty change with prednisone use or dose, perhaps because the dose of prednisone used was lower than in other studies. Our patient population used little alcohol and the duration of followup was not sufficient to show any fibrosis associated with alcohol use. At the time this study was begun, folic acid was not commonly used, nor was its importance realized. It was not included in our database and was not analyzed.

Our study included pre-MTX biopsies in 10 patients. Although it would have been desirable to obtain pre-MTX biopsies in all patients, this was not possible. Although this is regrettable, it is nevertheless helpful to have some baseline data for comparison to the followup data. Two biopsies were tested at baseline and at one and 2 years for MTX concentrations, and no change in MTX or polyglutamate concentration was found in repeated, within-biopsy measures of the MTX/polyglutamate concentrations in those specimens over the 2 year period. While it derives from only a very few biopsies, this finding was reassuring, in that the concentrations were stable in the samples over time. At the same time, we did not test stability to 3.5 years and we cannot comment regarding this aspect of the study, although we surmise that stability was likely to 3.5 years.

Table 5 indicates that some abnormalities are found in the liver of patients with RA not yet taking MTX, similar to others' findings in the literature. Most of our patients were taking NSAID and prednisone, although the NSAID had been withdrawn for at least 5 half-lives before the biopsies. Neither multiple linear regression nor logistic regression analysis showed an effect of NSAID on liver histology. There was a trend toward an effect of prednisone in the multiple linear regression, with increasing prednisone use associated with a trend toward fibrosis ($p = 0.065$), but this was not corroborated using logistic regression. It was not possible to say whether the observed histological abnormalities were due to RA or to the concomitant medications (without MTX) that were used. Using the Roenigk classification, Table 4 corroborates the data in Table 5. Although there was a trend to some increasing fibrosis with increasing duration of MTX dose (Table 4), this was never more than Roenigk Class IIIA and the findings were not statistically significant. The Iowa classification of liver histology and MTX use correlated more closely with measures of liver damage (AST, ALT) than the Roenigk classification (see Results). The Iowa classification seemed to reflect histologic evidence of liver damage (and lack of progression) as well as the Roenigk classification (see Results). The Iowa score is a reasonable way to measure the sensitivity of histological examination of liver pathology. It has face validity and seemed, in this small sample, to have construct validity, as it was more sensitive to change than the Roenigk scale. Further, it was clearly feasible and was reproducible (on reexamination by one investigator). The weakness of the present data with respect to the Iowa score rests on the small number of samples, the lack of use by more than one pathologist (FM), and the relatively small range of abnormalities found (Roenigk Class I to IIIA). The Iowa score seems a reasonable approach to pursue and may be useful in the future if it is further validated.

In our study, accumulation of high order polyglutamate was documented by the end of one year of therapy and it remained relatively stable through year 2. The relative decrease in these higher order polyglutamates at year 3.5 remains unexplained. These samples were stored at –70˚C for prolonged periods, but it is possible that some breakdown of higher order polyglutamates to the diglutamate occurred during the storage period of several years. Two

biopsies were tested at baseline and at one and 2 years, and no degradation of MTX or percentage of polyglutamates was found in this small sample. Breakdown beyond 2 years remains possible.

No clinically important correlations of MTX levels with histology, liver function tests, concomitant drug use, or disease activity were found. It appears that MTX is relatively safe within the time frame of our study, although the number of biopsies was small. At the same time, 2 patients did not enter the study because they wished not to undergo more liver biopsies after "traumatic baseline liver biopsies," pointing out that liver biopsies are not a trivial matter. If other data appear indicating the safety of MTX during similar time periods, one might consider revising the guidelines with respect to liver biopsies in patients with RA taking MTX. Perhaps larger numbers of patients followed for even longer periods would be needed to show such a relationship, but it was not possible for this patient cohort. The results imply that serum MTX concentrations are not likely to be useful measures of, or predictors for, histologic liver damage by MTX.

In summary, this analysis describes the accumulation and stabilization of MTX concentrations in the liver and examines correlations between MTX liver concentrations, patient demographics, liver histology, concomitant medications, and disease activity. No such correlations were found, decreasing the likelihood that MTX concentrations in serum would be useful measures to predict hepatotoxicity. This study also proposes a preliminary new histologic liver classification scheme, the Iowa score, which may be more sensitive to change than the Roenigk score.

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