Factors Associated with Myelosuppression Related to Low-Dose Methotrexate Therapy for Inflammatory Rheumatic Diseases

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Abstract

Objective

Severe myelosuppression is a serious concern in the management of rheumatic disease patients receiving methotrexate (MTX) therapy. This study was intended to explore factors associated with the development of MTX-related myelosuppression and its disease severity.

Methods

We retrospectively examined a total of 40 cases of MTX-related myelosuppression that had been filed in the registries of participating rheumatology and hematology divisions. Data before onset were compared with those of 120 controls matched for age and sex. Cytopenia was graded according to the National Cancer Institute criteria for adverse events. Data before and at onset were compared between the severe and non-severe groups.

Results

Non-use of folic acid supplements, concurrent medications, and low renal function were significantly associated with the development of myelosuppression (p < 0.001, p < 0.001, and p = 0.002, respectively). In addition, significantly lower MTX dosages, higher blood cell counts, and lower hemoglobin levels were seen in the myelosuppression group (p < 0.001). No patients exhibited leukocytopenia, neutropenia, or thrombocytopenia in routine blood monitoring taken within the past month. One-fourth developed myelosuppression within the first two months (an early-onset period). Myelosuppression was severe in approximately 40% of patients. Hypoalbuminemia and non-use of folic acid supplements were significantly
associated with the severity of pancytopenia ($p = 0.001$ and $0.008$, respectively). Besides these two factors, early onset and the use of lower doses of MTX were significantly associated with the severity of neutropenia ($p = 0.003$, $0.007$, $0.003$, and $0.002$, respectively).

**Conclusions**

Myelosuppression can occur abruptly at any time during low-dose MTX therapy, but severe neutropenia is more likely to occur in the early-onset period of this therapy. Contrary to our expectations, disease severity was not dependent on MTX doses. Serum albumin levels and folic acid supplementation are the important factors affecting the severity of MTX-related pancytopenia and neutropenia.

**Introduction**

Methotrexate (MTX) is widely recognized as a key conventional synthetic disease-modifying antirheumatic drug (DMARD) in the treatment of inflammatory rheumatic diseases, especially rheumatoid arthritis (RA). This drug continues to serve as an anchor drug among DMARDs, mainly based on the following characteristics: its proven efficacy both during monotherapy and in combination with steroids, other conventional systemic DMARDs, and biological DMARDs; the ability to increase the efficacy of biological DMARDs when used in combination; and the acceptable long-term safety profile [1–3]. The 2013 update of EULAR recommendations for the management of RA with synthetic and biological DMARDs did not change the previous recommendation that MTX should be part of the first strategy in patients with active RA [4].

Although MTX was originally developed for use in anti-cancer therapy as part of a high-dose regimen, low-dose MTX has proven to be highly effective in the treatment of RA. Multi-national evidence-based recommendations state that oral MTX be started at 10 to 15 mg/week, with an escalation of 5 mg every two to four weeks up to 20 to 30 mg/week, depending on clinical response and tolerability [5]. In Japan, an MTX dosage of up to 16 mg/week is currently approved. Low-dose MTX therapy is usually well tolerated in clinical practice, and most adverse events are moderate and resolve following drug discontinuation [6–9]. However, long-term clinical experience with MTX in the treatment of RA has taught us that MTX can induce severe, potentially life-threatening adverse events, especially opportunistic infections (*Pneumocystis jirovecii* pneumonia, mycobacterial infections, and viral reactivation such as *de novo* hepatitis B and disseminated herpes zoster infection, etc.), pulmonary toxicity, and myelosuppression [10–13].

MTX-related myelosuppression is estimated to occur in 2 to 10.2% of patients with inflammatory rheumatic diseases [14–17]. Neutropenia is encountered most often, but anemia and thrombocytopenia also occur [14, 16–18]. Neutropenia and pancytopenia developed in 1.4 to 7% and 0.3 to 2.1% of patients receiving low-doses of MTX, respectively [15–23]. Although severe or fatal cases of myelosuppression have been reported in this clinical context, it is not clear which factors contribute to disease severity. It is generally believed that the severity of acute adverse effects is related to dose and frequency of MTX administration; however, a recent study suggested that serum MTX concentrations did not correlate with the degree of either neutropenia or thrombocytopenia [24].

For a better understanding of possible factors associated with the development of MTX-related myelosuppression and its disease severity in daily practice for inflammatory rheumatic
Patients and Methods

Study design and patients

The following divisions for rheumatology or hematology registered all patients who were treated for hematological disorders, including myelosuppression, because they are required to prepare and disclose annual reports regarding exact numbers of cases for individual diseases: Department of Rheumatology, NHO Kumamoto Saishunsou National Hospital; Department of Hematology, NHO National Kumamoto Medical Center; Institute of Rheumatology, Zenjinkai Shimin-no-Mori Hospital; Department of Hematology and Oncology, Kumamoto City Hospital; Yoshitama Clinic for Rheumatic Diseases; Clinical Research Center, NHO National Nagasaki Medical Center; and Rheumatic and Collagen Disease Center, Sasebo Chuo Hospital. By using the terms of myelosuppression, MTX, and rheumatic diseases, we searched these registry data and captured patients (of 18 years and over) who had been treated for myelosuppression related to MTX therapy for rheumatic diseases, and we then identified and enrolled eligible patients who fulfilled the following inclusion and exclusion criteria for this study. Myelosuppression was defined as at least one of the following conditions: leukopenia (leukocyte count below 3500/mm$^3$), thrombocytopenia (platelet count below 130000/mm$^3$), or anemia (hemoglobin level less than 11.5 g/dl for females or 13.5 g/dl for males). Since it is well known that patients with chronic inflammatory diseases such as RA often suffer from anemia [25, 26], we included a decrease of 0.5 g/dl or more than the latest hemoglobin values measured within one month to the definition criteria for newly developed anemia during MTX therapy. Patients with other possible causes known to be associated with myelosuppression were excluded from this study. As for the age- and sex-matched controls, we selected 120 rheumatic disease patients who had received MTX therapy in the participating divisions but had not developed myelosuppression.

From among all eligible patients, we identified patients who were diagnosed with pancytopenia, defined as the presence of leukopenia, thrombocytopenia, and anemia. Severe pancytopenia was defined as a leukocyte count below 2000/mm$^3$, a platelet count below 50000/mm$^3$, and a hemoglobin level less than 10.0 g/dl [22]. We also identified patients who were diagnosed with neutropenia, which was defined as a neutrophil count below 2000/mm$^3$. Severe neutropenia (agranulocytosis) was defined as a decrease in a neutrophil count to less than 500/mm$^3$ [27]. Patients’ cytopenia and hypoalbuminemia were graded according to the severity grade of the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.0 (http://ctep.cancer.gov).

Through reviewing patients’ medical records, we scrutinized demographic data, laboratory results before and at onset of myelosuppression, indications for MTX therapy, MTX dosages and duration, medications concurrently used during MTX therapy, preceding episodes of dehydration, folic acid supplementation, and clinical presentation at onset. The data were compared between the severe and non-sever patient groups.

This study was conducted in accordance with the principles of the Declaration of Helsinki (2008). The protocol of this study also meets the requirements of the Ethical Guidelines for Medical and Health Research Involving Human Subjects, Japan (2014) and has been approved...
Renal function
An estimated glomerular filtration rate (eGFR) was first calculated according to the following equation that had been developed for Japanese patients and was officially approved by the Japanese Society of Nephrology: 
\[ eGFR (\text{ml/min/1.73 m}^2) = 194 \times (\text{serum creatinine [mg/dl]})^{-1.094} \times (\text{age})^{-0.287} \times 0.739 \text{ (if female)} \]  
and was then corrected for each patient’s BSA. Corrected eGFR = GFR × BSA / 1.73. Renal insufficiency was defined as a corrected eGFR < 60 ml/min. eGFR categories were determined according to the Kidney Disease Improving Global Outcomes (KDIGO 2012) guidelines.

Statistical analysis
Statistical analyses were performed using the independent-measures t-test for comparisons of continuous variables between two groups. The chi-square test using 2 × 2 contingency tables was used for categorical variables. If cell values were less than 5, Fisher’s exact probability test was used. The Pearson’s correlation technique was used to investigate the degree of the relationship between weekly MTX doses prescribed and corrected eGFR values before onset of myelosuppression. For all tests, probability values (p values) < 0.01 were considered to indicate statistical significance. All calculations were performed using PASW Statistics version 22 (SPSS Japan Inc., Tokyo, Japan).

Results
Clinical and laboratory features of patients who developed myelosuppression during low-dose MTX therapy
Through searching the registries of the participating divisions, we identified a total of 40 patients who had developed myelosuppression during low-dose MTX therapy for inflammatory rheumatic disease between February 2005 and November 2014 (Table 1). Thirty-nine cases occurred in patients with RA and one in a patient who had been diagnosed with polymyalgia rheumatica (PMR). More than 90% of patients were 65 years of age or more. When compared with the age- and sex-matched control group, rates of folic acid supplementation were significantly lower in patients who had developed myelosuppression (cases versus controls: 42.5% versus 100%, p < 0.001). Corrected eGFR values before onset were also significantly lower in the case group (46.7 ml/min versus 56.3 ml/min, p = 0.002). In the case group, more patients had received concomitant medications (97.5% versus 62.5%, p < 0.001), especially nonsteroidal anti-inflammatory drugs (NSAIDS, 72.5% versus 10%, p < 0.001), antacids (45% versus 15%, p < 0.001), and prednisolone (67.5% versus 12.5%, p < 0.001). Counts of blood cells, such as leukocytes, neutrophils, and thrombocytes, were significantly higher in the case group (p < 0.001). Hemoglobin levels were lower in the case group (10.6 mg/dl versus 12.3 mg/dl, p < 0.001). Weekly MTX doses were significantly lower in the case group (5.7 mg versus 7.3 mg, p < 0.001). There was a significant correlation between MTX doses prescribed and corrected eGFR values before onset in those patients who had received MTX therapy (r = 0.421, p < 0.001).

Eleven patients (27.5%) developed myelosuppression within the first two months of MTX therapy. Eighteen patients (45%) had a preceding episode that could have caused dehydration.
Table 1. Clinical and laboratory characteristics of patients who developed myelosuppression during low-dose MTX.

|                      | Case (n = 40) | Control§ (n = 120) | p     |
|----------------------|--------------|--------------------|-------|
| Age, years, mean (SD)| 73.0 (8.7)   | -                  | -     |
| > 65, patient number (%)| 37 (92.5)  | -                  | -     |
| Female/male, patient number | 34/6   | -                  | -     |
| Indication for MTX therapy, RA/PMR, patient number | 39/1 | 120/0 | -     |
| MTX therapy          |              |                    |       |
| Doses, mg/week, mean (SD) | 5.7 (2.0)  | 7.3 (2.1)          | <0.001|
| Treatment duration, months, mean (SD) | 44.5 (48.9) | 68.7 (49.2) | 0.008 |
| Early onset*, patient number (%) | 11 (27.5) | -                 | -     |
| Folic acid supplementation, patient number (%) | 17 (42.5) | 120 (100) | <0.001|
| Concurrent medications, patient number (%) | 39 (97.5) | 75 (62.5) | <0.001|
| NSAIDs               | 29 (72.5)   | 12 (10)            | <0.001|
| Other biological and systemic DMARDs | 12 (30)† | 32 (26.7)         | 0.68  |
| Antiarrhythmic drugs | 3 (7.5)     | 3 (2.5)            | 0.17  |
| ARB/ACE inhibitors   | 7 (17.5)    | 33 (27.5)          | 0.29  |
| Antacids             | 18 (45)     | 18 (15)            | <0.001|
| H2 blockers          | 6 (15)      | 1 (0.8)            | 0.001 |
| Proton pump inhibitors| 12 (30)    | 17 (14.2)          | 0.033 |
| Prednisolone         | 27 (67.5)   | 15 (12.5)          | <0.001|
| None                 | 1 (2.5)     | 45 (37.5)          | <0.001|
| BMI, kg/m², mean (SD) | 20.3 (2.5) | 24.1 (16.1)       | 0.14  |
| Laboratory data before onset‡ |              |                    |       |
| Leucocytes, /mm³, mean (SD) | 6741 (2324)| 5003 (1445)      | <0.001|
| Neutrophils, /mm³, mean (SD) | 4696 (2378)| 3264 (1378)     | <0.001|
| Thrombocytes, x 10⁶/mm³, mean (SD) | 25.3 (9.9) | 18.6 (4.8)       | <0.001|
| Hemoglobin, g/dl, mean (SD) | 10.6 (1.4) | 12.3 (1.6)       | <0.001|
| Corrected eGFR, ml/min, mean (SD) | 46.7 (21.6) | 56.3 (15.5)  | 0.002 |

*Defined as the development of pancytopenia within the first two months of MTX therapy.
†Other DMARDs included salazosulfapyridine (n = 8), tumor necrosis factor inhibitors (n = 3), and tocilizumab (n = 1).
‡The latest available data within one month before onset of myelosuppression.
§Controls were selected by individual matching for age and sex. MTX, methotrexate; RA, rheumatoid arthritis; PMR polymyalgia rheumatica; eGFR, estimated glomerular filtration rate; NSAIDs, nonsteroidal anti-inflammatory drugs; DMARDs, disease-modifying antirheumatic drugs; ARB, angiotensin receptor blockers; ACE, angiotensin converting enzyme; BMI, body mass index; SD, standard deviation.

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Factors Affecting Hematotoxicity of Low-Dose MTX Therapy

(anorexia, seven cases; stomatitis, four cases; flu, three cases; pyelonephritis, two cases; pharyngitis, one case; diarrhea, one case). The onset was abrupt: no patients exhibited leukocytopenia, neutropenia, or thrombocytopenia in routine blood monitoring taken within one month before onset.

Severity of myelosuppression related to low-dose MTX therapy for inflammatory rheumatic diseases

As shown in Table 2, among the 40 cases of myelosuppression related to low-dose MTX therapy, 31 cases (77.5%) were pancytopenia, 8 (20%) were bicytopenia (three cases, leukopenia and thrombocytopenia; three cases, leukopenia and anemia; two cases, thrombocytopenia and anemia), and 1 (2.5%) was isolated neutropenia. Severe pancytopenia was observed in 12 out of
Table 2. Severity of myelosuppression related to low-dose MTX therapy for inflammatory rheumatic diseases (n = 40).

| Type of myelosuppression | Patient number (%) |
|--------------------------|--------------------|
| Pancytopenia              | 31 (77.5)          |
| Severe pancytopenia       | 12 (38.7)          |
| Bicytopenia              | 8 (20)             |
| Isolated leukopenia       | 1 (2.5)            |
| Leucocytes, /mm$^3$, mean (SD) | 1757 (1424)       |
| Leucopenia, patient number (%) | 38 (95)          |
| Grade 1 ($\geq 3000$ and $<3500$) | 3 (7.9)          |
| Grade 2 ($\geq 2000$ and $<3000$) | 13 (34.2)         |
| Grade 3 ($\geq 1000$ and $<2000$) | 7 (18.4)          |
| Grade 4 ($<1000$)        | 15 (39.5)          |
| Neutrophils, /mm$^3$, mean (SD) | 972 (1272)        |
| Neutropenia, patient number (%) | 37 (92.5)         |
| Grade 1 ($\geq 1500$ and $<2000$) | 3 (8.1)           |
| Grade 2 ($\geq 1000$ and $<15000$) | 7 (18.9)          |
| Grade 3 ($\geq 500$ and $<1000$) | 11 (29.7)         |
| Grade 4 ($<500$)         | 16 (43.2)          |
| Thrombocytes, /mm$^3$, mean (SD) | $6.3 \times 10^4$ (5.5 x $10^4$) |
| Thrombocytopenia, patient number (%) | 36 (90)           |
| Grade 1 ($\geq 75000$ and $<130000$) | 9 (25)            |
| Grade 2 ($\geq 50000$ and $<75000$) | 8 (22.2)          |
| Grade 3 ($\geq 25000$ and $<50000$) | 5 (13.9)          |
| Grade 4 ($<25000$)        | 14 (38.9)          |
| Hemoglobin, g/dl, mean (SD) | 7.8 (2.4)         |
| Anemia, patient number (%) | 36 (90)           |
| Grade 1 ($\geq 10.0$ and $<11.5$ (for female) or 13.5 (for male) | 5 (13.9)          |
| Grade 2 ($\geq 8.0$ and $<10.0$) | 9 (25)            |
| Grade 3 ($\geq 6.5$ and $<8.0$) | 12 (33.3)         |
| Grade 4 ($<6.5$)         | 10 (27.8)          |
| Albumin, g/dl, mean (SD) | 2.9 (0.7)         |
| Hypoalbuminemia, patient number (%) | 38 (95)          |
| Grade 1 ($\geq 3.0$ and $<4.0$) | 17 (44.7)         |
| Grade 2 ($\geq 2.0$ and $<3.0$) | 17 (44.7)         |
| Grade 3 ($<2.0$)         | 4 (10.5)           |
| Corrected eGFR, ml/min, mean (SD) | 35.1 (21.1)       |
| eGFR categories          |                    |
| G1 ($\geq 90$) | 1 (2.5) |
| G2 ($\geq 60$ and $<90$) | 3 (7.5) |
| G3 ($\geq 30$ and $<60$) | 19 (47.5) |
| G4 ($\geq 15$ and $<30$) | 9 (22.5) |
| G5 ($<15$) | 8 (20) |
| Rapid exacerbation of renal function* , patient number (%) | 21 (52.5) |
| Hepatotoxicity† , patient number (%) | 5 (12.5) |
| Mucositis at onset, patient number (%) | 18 (45) |
| Pulmonary toxicity (interstitial pneumonia) (%) | 0 |
| Infectious diseases‡ , patient number (%) | 20 (50) |
| Sepsis, patient number (%) | 11 (27.5) |

(Continued)
31 patients (38.7%). Patients with a severity grade of 4 accounted for 39.5% of leukopenia, 38.9% of thrombocytopenia, and 27.8% of anemia cases. Thirty-seven patients (92.5%) developed neutropenia, and among these, 16 (43.2%) were diagnosed with severe neutropenia (corresponding to a severity grade of 4). In nine patients, neutrophil counts were below 100/mm³, and among those, five were found to have neutrophil counts of zero. Hypoalbuminemia and renal insufficiency were observed in the majority of myelosuppression patients (95% and 90%, respectively). Approximately half of the patients experienced a rapid exacerbation of renal function. Five patients had hepatotoxicity at the onset of myelosuppression, but no case progressed to liver failure. No patient showed pulmonary toxicity, namely interstitial pneumonia. Half of the cases were complicated by infectious diseases, and among these, 11 developed sepsis.

Three patients were under hemodialysis. All of the patients developed a severity grade of 4 for neutropenia and thrombocytopenia within one month. Weekly MTX doses prescribed were very small, ranging from 2 to 4 mg. After the introduction of MTX therapy, their renal function rapidly exacerbated.

Despite intensive treatment with intravenous antibiotics, leucovorin, granulocyte colony stimulating factor, and transfusions of blood products including packed red cell, platelets, and fresh frozen plasma, five patients died of sepsis. Among the fatal cases, four were severe pancytopenia with grade 4 neutropenia and thrombocytopenia. One case was bicytopenia with grade 3 anemia and grade 4 thrombocytopenia. This patient’s disease was complicated by disseminated intravascular coagulation. Two patients had received only a total of 4–6 mg of MTX (case 1, two doses of 2 mg; case 2, a single dose of 6 mg). One case (case 1) occurred in a patient under hemodialysis.

Factors affecting the severity of pancytopenia and neutropenia

As shown in Table 3, non-use of folic acid supplements and serum albumin levels were significantly associated with the severity of pancytopenia (non-severe versus severe: 57.9% versus 8.3%, \( p = 0.008 \); 3.1 g/dl versus 2.3 g/dl, \( p = 0.001 \), respectively). There were no differences in MTX doses prescribed for both groups. Patients with severe pancytopenia were more likely to develop infectious diseases (31.6% versus 83.3%, \( p = 0.009 \)) and sepsis (5.3% versus 75%, \( p < 0.001 \)). Fatal outcomes were seen only in patients with severe pancytopenia.

We also compared the data between patients who had developed severe neutropenia and those with non-severe neutropenia (Table 4). Like severe pancytopenia, non-use of supplemental folic acid and serum albumin levels were the significant factors affecting the disease severity (non-severe versus severe: 66.7% versus 18.8%, \( p = 0.007 \); 3.2 g/dl versus 2.5 g/dl, \( p = 0.003 \), respectively). In addition, severe neutropenia was more likely to occur within the first two months than was non-severe neutropenia (9.5% versus 56.3%, \( p = 0.003 \)). MTX doses were
significantly lower in severe neutropenia patients than in non-severe neutropenia patients (6.6 mg/week versus 4.6 mg/week, \( p = 0.002 \)). Complication of infectious diseases and sepsis were more often seen in severe neutropenia patients than in non-severe neutropenia patients (33.3% versus 75%, \( p = 0.02 \); 9.5% versus 50%, \( p = 0.009 \), respectively). All of the fatal cases were seen in severe neutropenia patients.

**Discussion**

In the present study, serum albumin levels were identified as the significant factor that affected the severity of pancytopenia and neutropenia. Hypoalbuminemia has been reported as the factor predisposing rheumatic disease patients to myelosuppression [20–22, 24, 29–31]. MTX is moderately bound to plasma proteins, mainly albumin, with the fraction bound ranging from 46.5 to 54% [32]. MTX unbound to albumin, but not the bound form, can enter cells via the reduced folate carrier, and then MTX is converted to polyglutamate derivatives, which enhances its intracellular retention [33]. Since the toxic effects of MTX are considered to depend on the intracellular drug concentration and exposure duration, the increase in extracellular concentrations of unbound MTX can contribute to the development of severe myelosuppression. The reduction of serum albumin levels would lead to an increase in unbound MTX. Although hepatic impairment is known to decrease albumin production, only 12.5% of patients in the present study showed hepatotoxicity at the time of development of myelosuppression.
and none developed liver failure. The chronic nature of RA may be involved in hypoalbuminemia seen in our patient population.

Myelosuppression occurs more commonly as a late complication of low-dose MTX therapy, but it can also occur in an early-onset period (within one to two months), independently of dosage, possibly as an idiosyncratic reaction to MTX. MTX-related myelosuppression is generally considered to result from toxicity due to drug effects on folate antagonism in bone marrow, but the idiosyncratic reaction appears to be an immunological or hypersensitive phenomenon, which is unpredictable and can result in significant morbidity and mortality [34–36]. In the present study, one-fourth of patients developed myelosuppression within the first two months. MTX doses were much lower in those patients who had developed myelosuppression. In particular, severe neutropenia was more frequently seen in patients treated with lower doses as well as during the early-onset period of MTX therapy. However, we cannot exclude the possibility that the association between the use of lower MTX doses and the development of myelosuppression was simply due to the therapeutic selection of using lower doses of MTX for patients with worse renal function, because there was a significant correlation between corrected eGFR values and MTX doses. In both of the early-onset and late-onset cases, the occurrence of myelosuppression was abrupt, and it was difficult to detect this complication in its early stages, even though monthly blood monitoring was performed.

MTX and its metabolites are primarily eliminated in the urine through glomerular filtration and active tubular secretion via specific transporters for organic anions folates, and a small part

### Table 4. Factors associated with severity of MTX-related neutropenia (n = 37).

| Factor                                      | Non-severe (n = 21) | Severe (n = 16) | p    |
|---------------------------------------------|---------------------|-----------------|------|
| Age, years, mean (SD)                       | 73.4 (8.9)          | 70.8 (8.3)      | 0.37 |
| Female/male, patient number                | 17/4                | 14/2            | 0.68 |
| MTX doses, mg/week, mean (SD)              | 6.6 (1.9)           | 4.6 (1.7)       | 0.002|
| Duration of MTX therapy, months, mean (SD) | 58.8 (53.9)         | 25.0 (37.6)     | 0.039|
| Early onset*, patient number (%)           | 2 (9.5)             | 9 (56.3)        | 0.003|
| Folic acid supplementation, patient number (%) | 14 (66.7)         | 3 (18.8)        | 0.007|
| Concurrent medications, patient number (%) | 20 (95.2)           | 15 (93.8)       | 1.00 |
| BMI, kg/m², mean (SD)                      | 20.5 (2.3)          | 19.9 (2.7)      | 0.44 |
| Preceding dehydration, patient number (%)  | 7 (33.3)            | 10 (62.5)       | 0.078|
| Albumin at onset g/dl, mean (SD)           | 3.2 (0.6)           | 2.5 (0.7)       | 0.003|
| ≤3.0 g/dl at onset, patient number (%)     | 7 (33.3)            | 13 (81.3)       | 0.007|
| Corrected eGFR, ml/min, mean (SD)          |                     |                 |      |
| Before onset                                | 48.6 (14.3)         | 42.6 (30.0)     | 0.46 |
| At onset                                    | 39.7 (21.3)         | 29.3 (21.3)     | 0.15 |
| Rapid exacerbation of renal function†, patient number (%) | 8 (38.1)          | 11 (68.8)       | 0.065|
| Hepatotoxicity at onset‡, patient number (%) | 3 (14.3)             | 2 (12.5)        | 1.00 |
| Mucositis at onset, patient number (%)      | 6 (28.6)            | 10 (62.5)       | 0.039|
| Infectious diseases at onset, patient number (%) | 7 (33.3)           | 12 (75)         | 0.020|
| Complicated sepsis, patient number (%)      | 2 (9.5)             | 8 (50)          | 0.009|
| Fatal outcome, patient number (%)           | 0                   | 4 (25)          | 0.028|

*Defined as development of neutropenia within the first two months of MTX therapy.
†Defined as an increase of 0.3 mg/dl or more or a twofold increase or more in serum creatinine levels compared with the latest available data within one month before onset of neutropenia.
‡Defined as an at least twofold elevation in serum alanine aminotransferase and aspartate aminotransferase levels over the upper limit of normal. MTX, methotrexate; BMI, body mass index; eGFR, estimated glomerular filtration rate; SD, standard deviation.

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and none developed liver failure. The chronic nature of RA may be involved in hypoalbuminemia seen in our patient population.

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MTX and its metabolites are primarily eliminated in the urine through glomerular filtration and active tubular secretion via specific transporters for organic anions folates, and a small part
is excreted through the biliary tract. Thus, the contribution of renal secretion is greater than that of the hepatic mechanism [32, 37]. Slow elimination of MTX in patients with renal insufficiency leads to prolonged exposure of bone marrow tissues to this drug. Renal insufficiency has been incriminated as the major risk factor for myelosuppression [20–22, 24, 29, 30, 38, 39], and our recent real-life registered study for RA showed that approximately one-fourth of patients have renal insufficiency [26]. In the present study, corrected eGFR was significantly associated with the development of myelosuppression during low-dose MTX therapy, but it was less likely to be the significant factor associated with the severity of pancytopenia or neutropenia.

Interactions with antirheumatic drugs commonly administered with MTX, such as NSAIDs, prednisolone, and other DMARDs, may influence MTX toxicity [40]. In most previous case reports of MTX-related pancytopenia, patients had received multiple medications concomitantly [20–22, 24, 29, 38]. Excretion of MTX can be inhibited by the interaction with NSAIDs through several mechanisms, including decreased renal blood flow and glomerular filtration as well as competitive inhibition of active tubular secretion of MTX. In addition, almost all NSAIDs are more than 90% bound to plasma proteins, which can lead to competition with MTX for binding sites on albumin [41]. Through a systemic review of studies examining concurrent use of MTX and NSAIDs, however, Colebatch et al. showed that there was no increase in the rates of MTX withdrawal or major toxic reactions in the management of RA [42]. Only one study reported a correlation between transient thrombocytopenia and concurrent use with NSAIDs [43]. There are no definitive data published regarding whether there are possible drug interactions between prednisolone and MTX [44]. Concerning other DMARDs, sulfasalazine is known to inhibit active tubular excretion of MTX via the specific transporters [45], but it is not clear whether this interaction is clinically significant in hematological toxicity. Concurrent use of MTX with leflunomide is also reported to increase the risk of pancytopenia [46]. Regarding pharmacokinetic interactions between MTX and biological DMARDs, very few data have been published [44]. In the present study, we showed that concurrent medications, especially NSAIDs, antacids, and prednisolone, were the significant factors associated with the development of myelosuppression. There is the possibility that myelosuppression may have occurred more often in rheumatic disease patients whose disease activity was not well controlled. Higher blood cell counts (leukocytes, neutrophils, and thrombocytes) and lower hemoglobin levels were seen in the myelosuppression group, which may also suggest this possibility.

One of the main limitations of the present study is its small sample size, which prevented us to draw a more definitive conclusion. Nevertheless, our cohort of 40 patients is larger than that of any of the past studies in the literature. Second, we did not know, as the denominator, the exact number of patients who had received MTX because this study included patients who had started MTX therapy in other hospitals. Therefore it was impossible to calculate a true prevalence of myelosuppression related to this therapy. Third, the present study was performed retrospectively, which may confer certain inherent limitations, such as bias and confounding, on the study. However, since pancytopenia and neutropenia are rare complications in rheumatic disease patients receiving low-dose MTX [15–23], it was difficult to perform a prospective registry study, as the registry size required was too large to be feasible.

**Conclusions**

Serum albumin levels and folic acid supplementation were significantly associated with the severity of pancytopenia and neutropenia. Although low renal function and concurrent medications were the significant risk factors for the development of myelosuppression, they were less potent in affecting the disease severity. Myelosuppression occurred abruptly at any time
during low-dose MTX therapy, but severe neutropenia was more often seen in the early-onset period of this therapy. Contrary to our expectations, disease severity was not dose-dependent in low-dose MTX therapy. We should keep in mind that the early detection of myelosuppression is a challenging task, even when monthly blood monitoring is adequately performed during low-dose MTX therapy.

Author Contributions
Conceived and designed the experiments: SM MH YU. Performed the experiments: SM MH TK TH HT TY KM YU. Analyzed the data: SM MH TK TH HT TY KM YU. Contributed reagents/materials/analysis tools: SM MH TK TH HT TY KM YU. Wrote the paper: SM MH TK TH HT TY KM YU.

References
1. Pincus T, Yazici Y, Sokka T, Aletaha D, Smolen JS. Methotrexate as the "anchor drug" for the treatment of early rheumatoid arthritis. Clin Exp Rheumatol. 2003; 21(Suppl 31):S179–185. PMID:14969073
2. Jurgens MS, Jacobs JW, Bijlsma JW. The use of conventional disease-modifying anti-rheumatic drugs in established RA. Best Pract Res Clin Rheumatol. 2011; 25(4):523–533. doi:10.1016/j.berh.2011.10.006 PMID:22137922
3. Jacobs JW. Optimal use of non-biologic therapy in the treatment of rheumatoid arthritis. Rheumatology (Oxford). 2012; 51 Suppl 4:iv3–8.
4. Smolen JS, Landewe R, Breedveld FC, Buch M, Burmester G, Dougados M, et al. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying anti-rheumatic drugs: 2013 update. Ann Rheum Dis. 2014; 73(3):492–509. doi:10.1136/annrheumdis-2013-204573 PMID:24161836
5. Visser K, Katchamart W, Loza E, Martinez-Lopez JA, Salliot C, Trudeau J, et al. Multinational evidence-based recommendations for the use of methotrexate in rheumatic disorders with a focus on rheumatoid arthritis: Integrating systematic literature research and expert opinion of a broad international panel of rheumatologists in the 3E Initiative. Ann Rheum Dis. 2009; 68(7):1086–1093. doi: 10.1136/ard.2008. 094474 PMID: 19033291
6. Yazici Y. Methotrexate induced pancytopenia is rare and concern for it should not limit its use. Rheumatology (Oxford). 2006; 45 Suppl 1:iv3–8.
7. Salliot C, van der Heijde D. Long-term safety of methotrexate monotherapy in patients with rheumatoid arthritis: a systematic literature research. Ann Rheum Dis. 2009; 68(7):1100–1104. doi: 10.1136/ard. 2008.093690 PMID: 19060002
8. Katchamart W, Trudeau J, Phumethum V, Bombardier C. Efficacy and toxicity of methotrexate (MTX) monotherapy versus MTX combination therapy with non-biological disease-modifying anti-rheumatic drugs in rheumatoid arthritis: a systematic review and meta-analysis. Ann Rheum Dis. 2009; 68(7):1105–1112. doi: 10.1136/ard.2008.099861 PMID: 19054823
9. Varatharajan N, Lim IG, Anandacoomarasamy A, Russo R, Byth K, Spencer DG, et al. Methotrexate: long-term safety and efficacy in an Australian consultant rheumatology practice. Intern Med J. 2009; 39(4):228–236. doi: 10.1111/j.1445-5994.2009.01800.x PMID: 19402861
10. Kanik KS, Cash JM. Does methotrexate increase the risk of infection or malignancy? Rheum Dis Clin North Am. 1997; 23(4):955–967. PMID: 9361163
11. Lateef O, Shakoor N, Baik RA. Methotrexate pulmonary toxicity. Expert Opin Drug Saf. 2005; 4(4):723–730. PMID: 16011450
12. Mori S, Sugimoto M. Pneumocystis jirovecii infection: an emerging threat to patients with rheumatoid arthritis. Rheumatology (Oxford). 2012; 51(12):2120–2130.
13. Mori S, Fujiyama S. Hepatitis B virus reactivation associated with antirheumatic therapy: Risk and prophylaxis recommendations. World J Gastroenterol. 2015; 21(36):10274–10289. doi: 10.3748/wjg.v21.i36.10274 PMID: 26420955
14. Weinblatt ME. Toxicity of low dose methotrexate in rheumatoid arthritis. J Rheumatol Suppl. 1985; 12 Suppl 12:35–39. PMID: 391775
15. Buchbinder R, Hall S, Sambrook PN, Champion GD, Harkness A, Lewis D, et al. Methotrexate therapy in rheumatoid arthritis: a life table review of 587 patients treated in community practice. J Rheumatol. 1993; 20(4):639–644. PMID: 8496857
16. Hanrahan PS, Scrivens GA, Russell AS. Prospective long term follow-up of methotrexate therapy in rheumatoid arthritis: toxicity, efficacy and radiological progression. Br J Rheumatol. 1989; 28(2):147–153. PMID: 2706419
17. Kinder AJ, Hassell AB, Brand J, Brownfield A, Grove M, Shadforth MF. The treatment of inflammatory arthritis with methotrexate in clinical practice: treatment duration and incidence of adverse drug reactions. Rheumatology (Oxford). 2005; 44(1):61–66.
18. Grove ML, Hassell AB, Hay EM, Shadforth MF. Adverse reactions to disease-modifying anti-rheumatic drugs in clinical practice. QJM. 2001; 94(6):309–319. PMID: 11391029
19. Williams HJ, Wilkens RF, Samuelson CO Jr., Alarcon GS, Guttadauria M, Yarboro C, et al. Comparison of low-dose oral pulse methotrexate and placebo in the treatment of rheumatoid arthritis. A controlled clinical trial. Arthritis Rheum. 1985; 28(7):721–730. PMID: 3893441
20. Gutierrez-Urena S, Molina JF, Garcia CO, Cuellar ML, Espinoza LR. Pancytopenia secondary to methotrexate therapy in rheumatoid arthritis. Arthritis Rheum. 1996; 39(2):272–276. PMID: 8849378
21. Ohosone Y, Okano Y, Kameda H, Hama N, Mimori T, Akizuki M, et al. Clinical characteristics related to methotrexate-induced pancytopenia. Clin Rheumatol. 1997; 16(3):321–323. PMID: 9184276
22. Lim AY, Gaffney K, Scott DG. Methotrexate-induced pancytopenia: serious and under-reported? Our experience of 25 cases in 5 years. Rheumatology (Oxford). 2005; 44(8):1051–1055.
23. Gilani ST, Khan DA, Khan FA, Ahmed M. Adverse effects of low dose methotrexate in rheumatoid arthritis patients. J Coll Physicians Surg Pak. 2012; 22(2):101–104. doi: 10.2012/JCPSP.101104 PMID: 22313647
24. Kivity S, Zafrir Y, Loebstein R, Pauzner R, Mouallem M, Mayan H. Clinical characteristics and risk factors for low dose methotrexate toxicity: a cohort of 28 patients. Autoimmun Rev. 2014; 13(11):1109–1113. doi: 10.1016/j.autrev.2014.08.027 PMID: 25172240
25. Wolfe F, Michaud K. Anemia and renal function in patients with rheumatoid arthritis. J Rheumatol. 2006; 33(8):1516–1522. PMID: 16881108
26. Mori S, Yoshitama T, Hidaka T, Hirakata N, Ueki Y. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis. 2009; 53(6):982–992. doi: 10.1053/j.ajkd.2008.12.034 PMID: 19339088
27. Andersohn F, Konzen C, Garbe E. Systematic review: agranulocytosis induced by nonchemotherapy drugs. Ann Intern Med. 2007; 146(9):657–665. PMID: 17470834
28. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, et al. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis. 2008; 53(6):982–992. doi: 10.1053/j.ajkd.2008.12.034 PMID: 19339088
29. MacKinnon SK, Starkebaum G, Wilkens RF. Pancytopenia associated with low dose pulse methotrexate in the treatment of rheumatoid arthritis. Semin Arthritis Rheum. 1985; 15(2):119–126. PMID: 3877983
30. Berthelot JM, Maugars Y, Hamidou M, Chiffoleau A, Barrier J, Grolleau JY, et al. Pancytopenia and severe cytopenia induced by low-dose methotrexate. Eight case-reports and a review of one hundred cases from the literature (with twenty-four deaths). Rev Rhum Engl Ed. 1995; 62(7–8):477–486. PMID: 8574610
31. Kent PD, Luthra HS, Michet C Jr. Risk factors for methotrexate-induced abnormal laboratory monitoring results in patients with rheumatoid arthritis. J Rheumatol. 2004; 31(9):1727–1731. PMID: 15338491
32. Inoue K, Yuasa H. Molecular basis for pharmacokinetics and pharmacodynamics of methotrexate in rheumatoid arthritis therapy. Drug Metab Pharmacokinet. 2014; 29(1):12–19. doi: 10.1002/art.24034 PMID: 18975321
33. Knowles SR, Uetrecht J, Shear NH. Idiosyncratic drug reactions: the reactive metabolite syndromes. Lancet. 2000; 356(9241):1587–1591. PMID: 11075787
34. Yoon KH, Ng SC. Early onset methotrexate-induced pancytopenia and response to G-CSF: a report of two cases. J Clin Rheumatol. 2001; 7(1):17–20. PMID: 17039083
35. Hocaoglu N, Altilla R, Osen F, Tuncok Y. Early-onset pancytopenia and skin ulcer following low-dose methotrexate therapy. Hum Exp Toxicol. 2008; 27(7):585–589. doi: 10.1177/0960327108094507 PMID: 18929735
36. Hillson JL, Furst DE. Pharmacology and pharmacokinetics of methotrexate in rheumatic disease. Practical issues in treatment and design. Rheum Dis Clin North Am. 1997; 23(4):757–778. PMID: 9361154
38. Kuitunen T, Malmstrom J, Palva E, Pettersson T. Pancytopenia induced by low-dose methotrexate. A study of the cases reported to the Finnish Adverse Drug Reaction Register From 1991 to 1999. Scand J Rheumatol. 2005; 34(3):238–241. PMID: 16134732

39. al-Awadhi A, Dale P, McKendry RJ. Pancytopenia associated with low dose methotrexate therapy. A regional survey. J Rheumatol. 1993; 20(7):1121–1125. PMID: 8371202

40. Bourre-Tessier J, Harauvi B. Methotrexate drug interactions in the treatment of rheumatoid arthritis: a systematic review. J Rheumatol. 2010; 37(7):1416–1421. doi: 10.3899/jrheum.090153 PMID: 20436072

41. Crofford LJ. Use of NSAIDs in treating patients with arthritis. Arthritis Res Ther. 2013; 15 Suppl 3:S2. doi: 10.1186/ar4174 PMID: 24267197

42. Colebatch AN, Marks JL, van der Heijde DM, Edwards CJ. Safety of nonsteroidal antiinflammatory drugs and/or paracetamol in people receiving methotrexate for inflammatory arthritis: a Cochrane systematic review. J Rheumatol Suppl. 2012; 90:62–73. doi: 10.3899/jrheum.120345 PMID: 22942332

43. Franck H, Rau R, Herborn G. Thrombocytopenia in patients with rheumatoid arthritis on long-term treatment with low dose methotrexate. Clin Rheumatol. 1996; 15(3):266–270. PMID: 8793258

44. Patane M, Cirico M, Chimirri S, Ursini F, Naty S, Grembiale RD, et al. Interactions among Low Dose of Methotrexate and Drugs Used in the Treatment of Rheumatoid Arthritis. Adv Pharmacol Sci. 2013; 2013:313858. doi: 10.1155/2013/313858 PMID: 23737767

45. Elsby R, Fox L, Stresser D, Layton M, Butters C, Sharma P, et al. In vitro risk assessment of AZD9056 perpetrating a transporter-mediated drug-drug interaction with methotrexate. Eur J Pharm Sci. 2011; 43 (1–2):41–49. doi: 10.1016/j.ejps.2011.03.006 PMID: 21440623

46. McEvon J, Purcell PM, Hill RL, Calcino LJ, Riley CG. The incidence of pancytopenia in patients taking leflunomide alone or with methotrexate. Pharmacoepidemiol Drug Saf. 2007; 16(1):65–73. PMID: 16634119