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Vitamin C and Ferric Gluconate Modulatory Effects on Methotrexate-Induced Cytotoxicity and Oxidative Stress

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Abstract

Methotrexate (MTX) is a common drug for inflammatory disorders, such as rheumatoid arthritis, although it has been associated with cytogenetic abnormalities, which suggest genotoxicity. This research evaluated the impact of vitamin C and ferric gluconate on micronucleus (MN) frequency and oxidative stress in patients receiving MTX. One hundred subjects were divided into four groups: MTX alone, MTX + vitamin C, MTX + ferric gluconate and combination adjunct therapy. Blood samples were taken at baseline and 8 weeks to measure MN and oxidative stress. Following treatment, the MTX group had increased MN, while the adjunct therapy groups, particularly the combined group had decreased MN. Oxidative stress analyses showed decreased MDA and increased GSH, SOD and CAT in the adjunct groups, especially the combined group ($p < 0.001$). Correlation studies suggested that the improved oxidative status was correlated with genomic stability. These findings suggest that vitamin C and ferric gluconate, particularly the combination, are associated with improved oxidative status and reduced chromosomal damage in patients receiving MTX.

Keywords: Methotrexate, Micronucleus, Genotoxicity, and Oxidative Stress.

1. Introduction

Methotrexate (MTX) is a folate antagonist drug used in several pathologies, most notably cancer and inflammatory conditions such as rheumatoid arthritis (RA). The medication effects have been well described on folate-dependent one-carbon metabolism and associated pathways that affect nucleotide synthesis and cell division. Nonetheless, its biology also presents significant clinical risks for the toxicity factors that may limit dose intensity, duration, and tolerability [1]. Clinical reviews highlight that MTX toxic effects do not involve a single organ and might include convergent pathways of oxidative stress, inflammation, and cell damage. These mechanistic domains may be non-distinct: inflammatory signals may enhance reactive oxygen species (ROS) production, redox imbalance can enhance DNA damage, and DNA damage may enhance inflammatory and response to stress [2].

It is based on these reasons that a research design incorporating both cytogenetic endpoints and biochemical redox markers would be more informative of the characterization of MTX-associated biological impact than clinical symptoms or routine laboratory parameters alone. One of the most practical cytogenetic endpoints is the formation of micronuclei (MN), which are small extranuclear bodies that have chromosomal fragments or entire chromosomes that are not incorporated into daughter nuclei during mitosis. For these reasons MN frequency is an expression of chromosomal breakage (clastogenicity) and/or chromosome loss (aneugenicity), which is a biologically meaningful expression of genomic instability [3, 4].

MTX-associated injury has been connected to the imbalance of redox and oxidative pathways in both experimental and clinical settings. The clinical reviews have outlined oxidative stress as a feature of MTX toxicity [5]. Vitamin C is an essential dietary component that serves as a powerful antioxidant that scavenges ROS. Several studies in various conditions have suggested the role of vitamin C in modulating oxidative stress, although the magnitude, direction, and effect on oxidative stress depend on dose, duration, pre-study oxidative status, and the choice of outcome [6, 7].

In MTX-treated populations, where perhaps both oxidative and inflammatory pathways are concurrently active, vitamin C is thus a reasonable adjuvant to consider. Ferrlecit (ferric gluconate complex) is a clinically available intravenous iron therapy used to treat anemia resulting from iron deficiency. Iron is the most common transition metal in our bodies, and it is a vital micronutrient required by numerous enzymes. As a result, it is essential for several biological functions, such as DNA synthesis and repair, cell cycle regulation, oxygen transport, and energy production [8]. The general objective of this study is to assess the effect of vitamin C and ferric gluconate given alone or in combination with the genomic stability and oxidative stress status of patients receiving MTX therapy.

2. Materials and Methods

2. 1 Study Design

This prospective experimental study was carried out to assess the effects of the vitamin C plus ferric gluconate on the frequency of MN and oxidative stress biomarkers in patients receiving MTX therapy. A total of 112 patients were screened and 100 patients met eligibility criteria and completed baseline sampling. Follow-up completion in week 8 was 96% (n=96), with four being lost because of relocation or missed appointments.

Eligible participants were adults from 18-65 years diagnosed with rheumatoid arthritis (RA). All patients had to be on MTX therapy given either orally or subcutaneously at a stable dose for at least four weeks before sampling at baseline. This stability criterion was essential to reduce the fluctuation of exposure of drugs that could affect the oxidative stress or genotoxicity markers.

2. 2 Grouping and Exposure Details

A total of 100 patients were divided into four exposure groups, each group consisted of 25 patients. Group 1 (G1) patients received MTX only and used as a standard of care control. The median dose at MTX was 15 mg/week (interquartile range [IQR] 10-20 mg), which reflects standard therapeutic practice in the management of RA. Group 2 (G2) patients received MTX and vitamin C. The patients received 500 mg daily oral vitamin C for a period of 8 weeks. Group 3 (G3) patients received MTX plus ferric gluconate. Patients were given Ferrlecit 125 mg per infusion.

A total of 2 to 4 infusions were given over a 2 to 4 weeks period depending on baseline ferritin levels, transferrin saturation (TSAT) and hemoglobin levels. The average total elemental iron supplied was 375 ± 125 mg. Group 4 (G4) was the group of patients that received the combined adjunctive regimen of MTX, vitamin C, and ferric gluconate.

2. 2. 1 Concomitant Medications

The use of non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and biologic disease-modifying antirheumatic drugs (DMARDs) was recorded and entered statistical analyses as a covariate. Low-dose prednisone (10 mg/day or less) was taken by 31% of the participants. Concomitant folic acid supplementation was reported in 92% of the participants.

The most frequent dose of folic acid was 5 mg weekly or an equivalent regimen, and the specific regimen was recorded to account for the potential modulation of toxicity of MTX.

2. 3 Sampling Schedule

Blood sampling was done on fixed timepoints to ensure standardization of outcome assessment. Baseline sampling (T0) was carried out at the time of enrollment, before vitamin C administration groups 2 and 4 and before the first infusion of Ferrlecit groups 3 and 4. Follow-up sampling (T1) was performed at week 8 (± 1 week) for all study groups. An additional optional time point (T2) at week 12 was obtained in a subset of participants (n = 34) for sensitivity analyses of temporal trends. However, T2 data were not included for primary endpoint testing.

2. 4 Sample Collection and Laboratory Methods

2. 4. 1 Blood Collection and Processing

At each point, 10 mL of venous blood was collected under aseptic conditions. From this, 4 mL was drawn into EDTA tubes for lymphocyte culture and micronucleus (MN) assay, and 6 mL was collected into serum tubes for oxidative stress biomarkers and routine laboratory analyses.

2. 4. 2 Micronucleus Assay

The cytokinesis-block micronucleus (CBMN) assay was used to measure the frequency of micronuclei in peripheral blood lymphocytes. Peripheral blood cultures in RPMI medium were supplemented with fetal bovine serum and phytohemagglutinin. The cells were cultured for 72 hours at 37°C and 5% CO_2. Easier to recognize binucleated cells, cytokinesis was blocked at 44 hours by adding cytochalasin B. Then, cells were smeared, fixed and stained using established protocols for Giemsa staining. For each participant and time point, 1000 binucleated cells were scored at 1000× magnification for standardized micronucleus scoring criteria. Results were expressed as micronuclei per 1000 binucleated cells.

2. 4. 3 Oxidative Stress Biomarkers

The serum oxidative stress biomarkers were measured with colorimetric and enzymatic assay kits that are valid according to the manufacturer's instructions. The parameters assessed were malondialdehyde (MDA, nmol/mL), reduced glutathione (GSH, micro-mol/L), superoxide dismutase (SOD, U/mL) and catalase (CAT, U/L).

2. 4. 4 Additional Clinical Laboratories (Covariates)

At baseline (T0) and week 8 (T1) routine laboratory parameters were obtained and recorded as covariates. These included complete blood count (hemoglobin, white blood cells, platelets), iron indices (ferritin and TSAT), inflammatory marker C-reactive protein (CRP) and safety parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum creatinine and calculated estimated glomerular filtration rate (eGFR).

2.5 Statistical Analysis

All statistical analyses were performed using the SPSS software package (version 26). Within-group changes from T0 to T1 were assessed by using paired statistical tests, which were chosen based on the distribution of data. For the normally distributed variables, paired t-tests were used while the Wilcoxon signed-rank test was used for non-parametric data. For primary between-group comparisons, analysis of covariance (ANCOVA) was used to compare the frequency of MNs at T1 between the study groups.

The model was adjusted for baseline values of MN as well as relevant covariates, such as age, sex, MTX dose, folic acid supplementation and C-reactive protein (CRP) levels, to obtain control for possible confounding. Secondary oxidative stress biomarkers were analyzed with the same modeling framework, both ANCOVA and mixed effects.

3. Results

3. 1 The baseline characteristics and participant influx

A total of 112 patients were screened, of whom 100 met the eligibility criteria and were recruited at baseline (T0). At week 8 (T1), 96 patients (96%) completed follow-up and were included in the primary analysis, as shown in figure 1. Baseline demographic, clinical, and laboratory characteristics of the study groups are summarized in table 1.

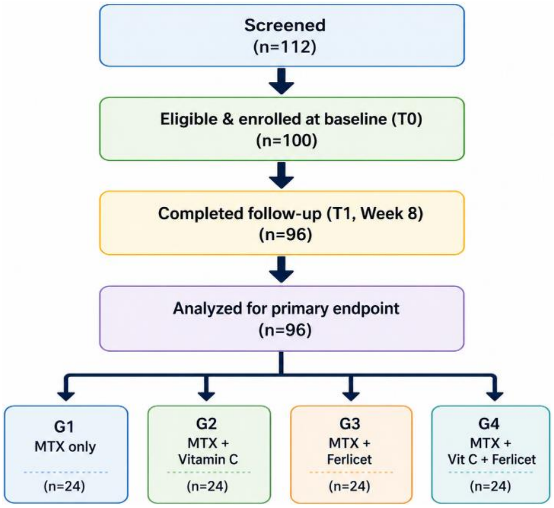

Figure 1: Patient enrollment and grouping flow.

Table 1: Baseline characteristics at T0 by study groups.

Variable	G1 MTX (n=25)	G2 MTX+ Vit C (n=25)	G3 MTX+ Ferrlecit (n=25)	G4 MTX +Vit C +Ferrlecit (n=25)
Age (years) mean ± SD	46.2 ± 9.1	44.8 ± 8.7	47.0 ± 9.4	45.6 ± 8.9
Female, n (%)	18 (72%)	17 (68%)	19 (76%)	18 (72%)
BMI (kg/m²), mean ± SD	27.3 ± 3.8	26.9 ± 4.1	27.8 ± 3.6	27.1 ± 3.9
RA duration (years), median (IQR)	6 (3–9)	5 (3–8)	7 (4–10)	6 (3–9)
MTX dose (mg/week), median (IQR)	15 (10–20)	15 (10–20)	15 (12.5–20)	15 (10–20)
MTX route (oral), n (%)	16 (64%)	15 (60%)	15 (60%)	16 (64%)
Folic acid use,n (%)	23 (92%)	24 (96%)	23 (92%)	22 (88%)
Prednisone use, n (%)	7 (28%)	8 (32%)	8 (32%)	8 (32%)
CRP (mg/L), median (IQR)	7.8 (4.2–12.6)	7.1 (3.9–11.8)	8.3 (4.5–13.1)	7.6 (4.0–12.2)
Hb (g/dL), mean ± SD	12.7 ± 1.1	12.8 ± 1.0	11.6 ± 1.2	11.5 ± 1.1
Ferritin (ng/mL), median (IQR)	68 (41–105)	71 (44–112)	22 (14–34)	21 (13–33)
TSAT (%), mean ± SD	21.4 ± 6.1	22.1 ± 6.4	13.2 ± 4.8	12.8 ± 4.6

3. 2 Primary Outcome: Micronucleus (MN) Frequency

At baseline (T0), the frequency of MN was found to be comparable across all study groups. Through week 8 (T1), an overall upward shift in MN frequency was observed in the MTX-only group, while adjunct treated groups showed reduction in MN distributions at follow-up, with the greatest downward shift in the combined vitamin C + Ferrlecit group as shown in figure 2. When analyzed as change scores (Δ MN = T1 – T0), the MTX-only group showed a net increase, and both the vitamin C and Ferrlecit groups showed net reductions. The combined group had a higher reduction with non-overlapping confidence intervals compared to MTX-only figure 3.

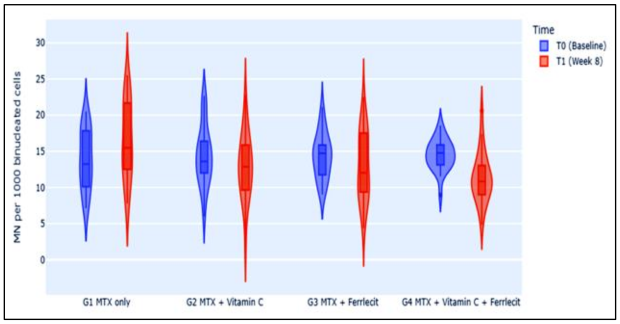

Figure 2: MN frequency at T0 and T1 by group.

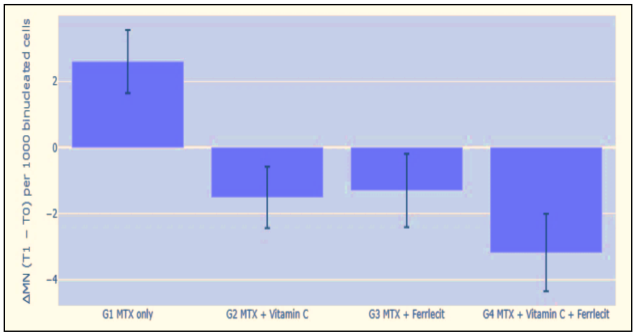

Figure 3: ΔMN by group with 95% confidence intervals.

In adjusted models (ANCOVA) for baseline MN, age, sex, MTX dose, folic acid use, and CRP, all the adjunct groups had significantly lower MN frequency at T1 than MTX-only. The results of the vitamin C + Ferrlecit group showed the greatest adjusted reduction (-5.79 MN/1000 binucleated cells, 95% CI -7.31 to -4.27, $p < 0.001$). Vitamin C alone and Ferrlecit alone also were linked to significant reductions table 2.

Table 2: Adjusted estimates for primary and secondary outcomes at T1 (vs MTX-only).

Group (vs G1)	**G1 MTX only**	**G2 MTX + Vit C**	**G3 MTX + Ferrlecit**	**G4 MTX + Vit C + Ferrlecit**
Adj. ΔMN at T1 (95% CI)	Reference	−4.08 (−5.64 to −2.51)	−3.72 (−5.29 to −2.14)	−5.79 (−7.31 to −4.27)
p	—	1.84e−06	1.28e−05	5.03e−11
Adj. ΔMDA at T1 (95% CI)	Reference	−1.12 (−1.38 to −0.87)	−0.89 (−1.15 to −0.64)	−1.30 (−1.55 to −1.05)
p	—	2.50e−13	1.07e−09	8.97e−17
Adj. ΔGSH at T1 (95% CI)	Reference	+1.70 (≈ +1.24 to +2.16)	+0.97 (≈ +0.52 to +1.43)	+1.79 (≈ +1.35 to +2.23)
p	—	1.81e−10	6.78e−05	4.71e−12
Adj. ΔSOD at T1 (95% CI)	Reference	+18.78 (13.30 to 24.26)	+18.20 (12.59 to 23.82)	+23.82 (18.60 to 29.03)
p	—	1.64e−09	8.41e−09	4.40e−14
Adj. ΔCAT at T1 (95% CI)	Reference	+10.10 (5.96 to 14.24)	+9.63 (5.46 to 13.81)	+13.76 (9.75 to 17.78)
p	—	6.71e−06	1.88e−05	1.63e−09

3. 3 Secondary Outcomes: Oxidative Stress Biomarkers

Changes in oxidative stress biomarkers from baseline to week 8 revealed a coherent change in biomarkers across adjunct groups in figure 4. Relative to MTX-only, adjunct therapies were linked to lower MDA (reduced lipid peroxidation) and higher antioxidant capacity (GSH, SOD and CAT). The combined group consistently showed the greatest beneficial shifts among biomarkers.

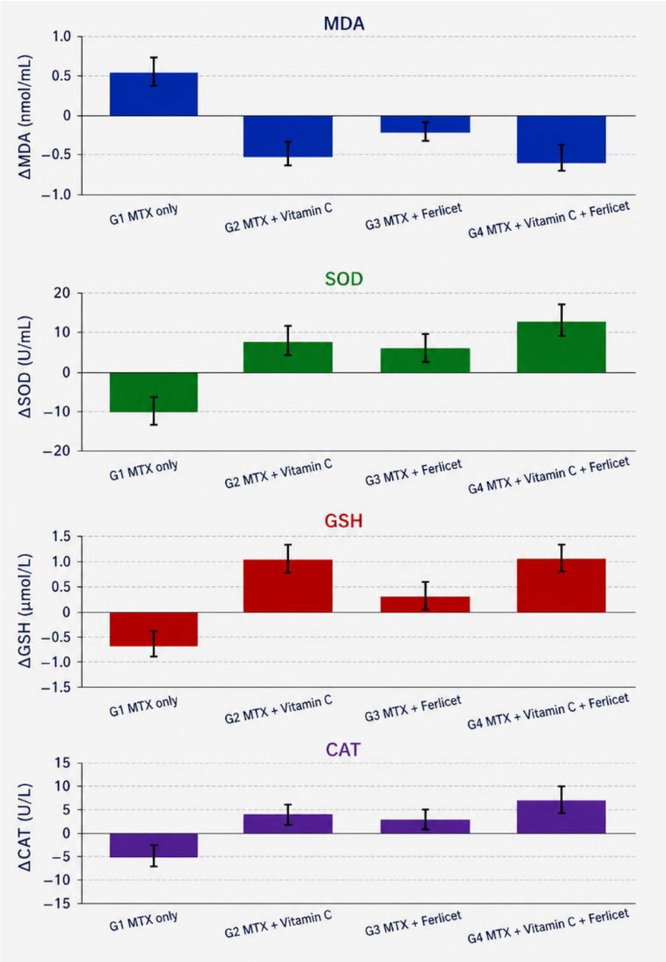

Figure 4: Changes in oxidative stress biomarkers (ΔMDA, ΔGSH, ΔSOD, ΔCAT) by group (mean ± 95% CI).

These findings were supported by adjusted analyses in table 2. Compared with MTX-only, the combined group had the greatest adjusted reduction in MDA (-1.30 nmol/mL, 95% CI, -1.55 to -1.05, $p < 0.001$) and the greatest adjusted improvements in GSH, SOD and CAT. Vitamin C alone also had strong improvements in the redox markers, and Ferrlecit alone had moderate but significant favorable improvements.

3. 4 Association Between Genomic and Oxidative Changes

Associations analysis suggested that the improvement in the oxidative status was in the same direction as the improvement in the genomic status (i.e., ΔMN tended to increase with increasing ΔMDA, and decrease with increasing ΔGSH). These associations are shown in figure 5 (interactive selection between ΔMDA and ΔGSH).

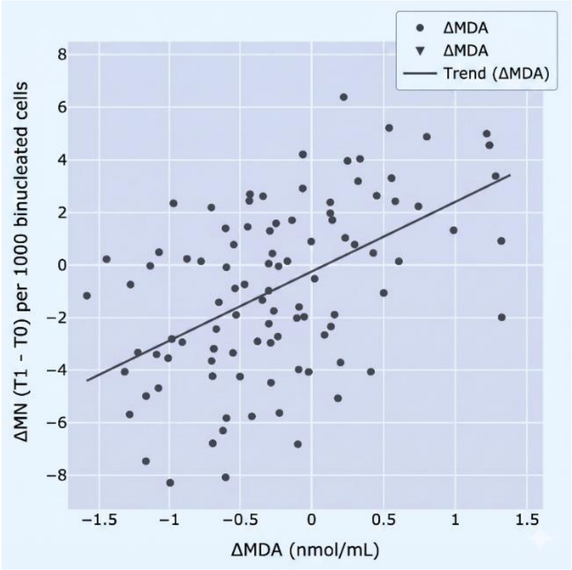

Figure 5: Relationship between ΔMN and changes in oxidative stress markers (ΔMDA or ΔGSH) with trendline.

4. Discussion

Results of the study indicate that the administration of vitamin C and ferric gluconate was associated with enhanced genomic stability and redox status in patients treated with MTX as reflected by a decrease in MN frequency and a concerted change in oxidative stress markers. These observations are consistent with the current hypothesis of mechanistic models of MTX toxicity that emphasise the role of the interaction between oxidative stress, inflammation and pathways of downstream DNA damage which may lead to the expression of chromosomal instability in the proliferating lymphocytes.

Recent clinical integration of MTX toxicity has shown oxidative stress to be a consistent underlying theme of toxicity across a number of systems, which justifies the simultaneous monitoring of cytogenetic and biochemical endpoints as opposed to clinical manifestations and conventional safety laboratory tests [2]. The ongoing inflammatory status in RA results in elevated reactive oxygen species (ROS) production and a relative decrease in antioxidant capacity, and consequently, a pro-oxidative environment. In this context, metabolic stress associated with treatment with MTX may further contribute to the induction of oxidative/genotoxic stress [9].

In this respect, results showing that the variation in MN frequency and oxidative biomarkers occurred in consistent and biologically plausible directions (such as positive tracking between the change in MN

(ΔMN) and oxidative biomarker (ΔMDA) and inverse relationships with the change in the antioxidant glutathione (ΔGSH)) add to the internal validity of the findings and suggest a link between redox changes in systemic circulation and cytogenetic responses. The evolution of the CBMN assay has further contributed to the enhanced interpretive value of the test by enabling both chromosomal instability and cytostasis or cytotoxicity indices to be measured to improve the level of detail offered by the test during exposure studies [10].

Recent methodological assessments have confirmed the robustness, reproducibility and suitability of the assay for comparative clinical research if standardized culture conditions and scoring criteria are strictly applied [11]. This is a context of validated methodology, in which the magnitude and the direction of the reduction of MNs (from the groups treated with adjuncts) is consistent with the idea that attenuation of the oxidative burden could be translated into measurable cytogenetic benefit. With regard to the biomarkers of oxidative stress, the decrease of MDA noted in the adjunctive groups is compatible with data in the literature on RA where serum MDA has been shown to be a valid marker of oxidative status and to correlate with other more general inflammatory and metabolic features. Large, well-characterized analyses of RA cohorts have provided clinical prognostic interpretability to MDA as a meaningful (oxidative) biomarker [12].

More recent clinical studies also support the usefulness of MDA as reflecting the burden of oxidative stress and a potential relationship with measures of disease activity, imparting external validity for interpreting the reduction in MDA as a favourable biological shift [13]. The simultaneous increase in antioxidant defenses (GSH, SOD and CAT) further argues in favor of the possibility that these changes are indicative of an actual redox improvement, and not random variation.

The improvements seen in vitamin C exposed groups are consistent with contemporary reviews of vitamin C as a redox-active micronutrient with antioxidant and immuno-modulatory components relevant to autoimmune illnesses such as RA. These analyses suggest the role of vitamin C in modulating oxidative stress and inflammatory microenvironment conditions, both of which may potentially result in decreased oxidative DNA damage and indirectly to diminished responses of chromosomal instability [14]. The interpretation of Ferrlecit® exposure is more complex due to the double-edged nature of iron. While iron repletion can improve the hematologic parameters as well as correct the iron-restricted erythropoiesis, iron also interacts with redox processes depending on labile iron pool and inflammatory context. Contemporary reviews of intravenous iron therapy focus on formulation-specific pharmacologic characteristics and call for empirical assessment of oxidative and inflammatory consequences as opposed to presumptive, uniform pro-oxidant effects for compounds and dosing strategies [15].

Our finding that exposure to Ferrlecit® was not linked to deterioration of oxidative stress markers and rather showed improvements is consistent with clinical evidence from iron-deficient populations showing that intravenous iron did not necessarily result in oxidative stress under controlled therapeutic conditions. Randomized pilot studies comparing various intravenous iron formulations in chronic kidney disease and iron deficiency have reported no induction of oxidative stress and inflammatory biomarkers after administration [16].

Notably, the most consistent and significant positive changes with MN frequency and oxidative biomarkers were noted in the combined vitamin C and Ferrlecit® group. Nevertheless, since Ferrlecit® exposure in routine clinical practice is identified by indication, and not by random allocation, residual confounding, particularly indication bias, cannot be totally ruled out although multivariable adjustment was done. Future studies using propensity score methodologies or randomized designs in eligible populations of iron-deficient MTX-treated patients would enhance causality.

5. Conclusion

The current study findings suggest that vitamin C and ferric gluconate, particularly in combination, are associated with improved genomic stability and redox balance in MTX-treated patients. Results also further demonstrate the clinical relevance of combining the assessment of MN frequency with a multi-marker oxidative stress panel for monitoring genomic stability and redox status in clinical research settings and therefore provide a methodologically robust approach.

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