



Research Article

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Mechanistic Divergence in Chemotherapy-Induced Genotoxicity: A Comparative *in vivo* Assessment of Different Antineoplastic Drug Classes in Murine Bone Marrow and Spleen

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Abstract

Background: Cancer chemotherapeutic agents, while effective against malignant cells, can also damage rapidly dividing normal tissues such as the bone marrow, raising concern about their genotoxic potential. **Objective:** To compare the acute *in vivo* cytotoxic and genotoxic effects of four mechanistically distinct antineoplastic agents on the bone marrow and splenic cells of albino mice. **Methods:** Sixty albino mice were randomly allocated to five groups (n=12): a negative control receiving distilled water and four groups receiving a single intraperitoneal dose of cisplatin (12 mg/kg), methotrexate (20 mg/kg), doxorubicin (5 mg/kg), or cyclophosphamide (50 mg/kg). Twenty-four hours after treatment, bone marrow and spleen samples were evaluated for mitotic index (MI), total chromosomal aberrations (TCA), and micronucleus (MN) frequency. **Results:** All four agents significantly reduced MI and increased TCA and MN frequency in both tissues compared with the control ($p < 0.05$). Methotrexate produced the smallest changes across all endpoints, whereas cyclophosphamide and cisplatin produced moderate, distinct effects. Doxorubicin caused the greatest reduction in MI and the highest TCA and MN frequencies. **Conclusions:** Genotoxicity ranked doxorubicin > cyclophosphamide > cisplatin > methotrexate, inversely reflecting the antiproliferative potency of these agents and paralleling the hematological toxicity observed clinically.

Keywords: Antineoplastic agents; Chromosome aberrations; Genotoxicity; Micronucleus tests; Mitotic index.

الاختلاف الميكانيكي في السمية الجينية الناتجة عن العلاج الكيميائي: تقييم مقارن في الجسم الحي لفئات مختلفة من أدوية مضادة للأورام في نخاع العظم وطحال الفئران

الخلاصة

الخلفية: العوامل الكيميائية العلاجية للسرطان، رغم فعاليتها ضد الخلايا الخبيثة، يمكن أن تتلف الأنسجة الطبيعية سريعة النمو مثل نخاع العظم، مما يؤثر القلق بشأن إمكانية التسبب الجيني. **الهدف:** مقارنة التأثيرات السمية الحادة للخلايا لأربعة عوامل مضادة للأورام تعمل بميكانيكية مختلفة على نخاع العظم وخلايا الطحال في الفئران البيضاء. **الطرائق:** تم تقسيم ستين فأراً أبيض عشوائياً إلى خمس مجموعات (n=12): مجموعة ضابطة سلبية تتلقى ماء مقطراً وأربع مجموعات تلقت جرعة واحدة داخل الصفاق من السيسبلائين (12 ملغ/كغ)، الميتوتركسيت (20 ملغ/كغ)، دوكسوروبيسين (5 ملغ/كغ)، أو السيكلوفوسفاميد (50 ملغ/كغ). بعد أربع وعشرين ساعة من العلاج، تم فحص عينات نخاع العظم والطحال للكشف عن مؤشر الانقسام (MI)، والانحرافات الكلي الكروموسومي (TCA)، وتكرار النوى الدقيقة (MN). **النتائج:** جميع العوامل الأربعة قللت بشكل ملحوظ من MI وزادت من تردد TCA و MN في كلا النسيجين مقارنة بالمجموعة الضابطة ($p < 0.05$). تسبب الميتوتركسيت في أصغر التغيرات عبر جميع الأطراف النهائية، بينما تسبب السيكلوفوسفاميد والسيسبلائين في تأثيرات متوسطة ومميزة. تسبب دوكسوروبيسين في أكبر انخفاض في MI وأعلى ترددات TCA و MN. **الاستنتاجات:** صنفت السمية الجينية دوكسوروبيسين > السيكلوفوسفاميد > سيسبلائين > الميتوتركسيت، مما يؤثر عكسياً قوة مضادة للتكاثر لهذه العوامل ويوازي السمية الدموية التي لوحظت سريرياً.

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INTRODUCTION

Genotoxicity is the property of some substances to damage genetic material in a cell. This can occur directly, by interacting with the DNA, or indirectly, by interrupting the processes that help DNA replicate, repair itself, or segregate during cell division [1]. Damage to a cell's genetic material can have major repercussions, because it alters the way in which cells typically divide, grow, and function. Damage that is not correctly repaired can cause permanent alterations in cellular DNA, and these changes can be passed on to subsequent cell generations, increasing the risk of cancer, birth abnormalities, and premature aging [2-5].

Genotoxicity therefore has a far-reaching impact, not only at the cellular level but also at the level of overall health. Understanding the mechanisms of genotoxic agents and their mitigation is important for designing strategies that limit cellular harm and its downstream health consequences. At the molecular level, genotoxic processes include base and nucleotide modification, single- and double-strand breaks, DNA-protein and DNA-DNA cross-links, and bulky adducts that distort the double helix and stall replication forks. Cells counteract these lesions through coordinated repair pathways such as base excision repair, nucleotide excision repair, and double-strand break repair; when the burden of damage exceeds repair capacity, genomic

instability results [6,7]. Cytogenetically, this instability manifests as structural and numerical chromosomal aberrations, disruption of the mitotic spindle and cell cycle checkpoints, and the appearance of micronuclei—small extranuclear bodies composed of acentric fragments or whole chromosomes that fail to segregate correctly during anaphase [8-10]. Methotrexate, cyclophosphamide, cisplatin, and doxorubicin are among the most widely used agents in oncology and represent four mechanistically distinct classes of cytotoxic drugs, each with a well-characterized but uniquely mediated genotoxic profile [11,12]. Methotrexate is a folate analogue that competitively inhibits dihydrofolate reductase, preventing regeneration of tetrahydrofolate required for *de novo* purine and thymidylate synthesis; this depletes nucleotide precursors and impairs DNA synthesis and repair in rapidly dividing cells, underlying both its antineoplastic activity and its capacity to induce genomic lesions when repair substrates become limiting [13,14]. This antifolate action has been linked experimentally to DNA damage and senescence induction in endothelial cells, and although methotrexate has comparatively mild genotoxic activity relative to DNA cross-linking agents, its capacity to induce genomic damage remains quantifiable and is thought to be mediated predominantly through reactive-oxygen-species (ROS)-dependent pathways [15,16]. Cyclophosphamide, by contrast, is a prodrug of the nitrogen mustard family that requires bioactivation by hepatic cytochrome P450 to phosphoramidate mustard, the major alkylating metabolite, which covalently cross-links interstrand and intrastrand DNA at the N7 position of guanine, directly interfering with replication and transcription and triggering p53-dependent apoptosis [17,18]. Cytokinesis-block micronucleus and chromosomal aberration assays in human lymphocytes and *in vivo* rodent models have confirmed cyclophosphamide-induced, dose-dependent increases in micronucleus frequency, chromosomal breakage, oxidative DNA lesions such as 8-hydroxy-2'-deoxyguanosine, and measurable γ H2AX induction in peripheral tissues [19,20]. Cisplatin, a platinum-based coordination complex, follows a different chemical route: following cellular uptake and intracellular aquation, it mainly forms 1,2-intrastrand cross-links with purine bases, bending and unwinding the DNA helix in a manner that blocks replication and transcription and ultimately activates apoptotic pathways [21,22]. The precise downstream consequences of cisplatin-DNA adduct processing remain incompletely understood after four decades of clinical use, and ongoing work continues to refine current understanding of how platinum-induced lesions translate into cytogenetic damage [23]. Doxorubicin, an anthracycline antibiotic, exerts cytotoxicity through two mechanisms: intercalation between DNA base pairs, which alters chromatin structure and causes strand

cleavage [24], and poisoning of topoisomerase II, stabilizing an otherwise transient cleavage complex and converting it into persistent double-strand breaks [24]. This topoisomerase-mediated damage, together with the redox cycling and ROS production generated by doxorubicin, accounts for its pronounced genotoxic and cardiotoxic character even at therapeutically standard dosages [25]. The present investigation was carried out to compare the genotoxic effects of methotrexate, cisplatin, cyclophosphamide, and doxorubicin on the bone marrow and splenic cells of mice. Although the genotoxic effects of these four agents have each been reported individually in previous studies, they have rarely been evaluated concurrently, under an identical experimental protocol, dosing schedule, and observation time point, in the same animal model. The novelty of the present work lies in this direct, within-study comparison of four mechanistically distinct antineoplastic drug classes across three complementary endpoints (mitotic index, chromosomal aberrations, and micronucleus frequency) in two tissues simultaneously—bone marrow, the principal hematopoietic compartment, and spleen, a secondary lymphoid organ with a distinct proliferative and immunological profile—which allows a relative genotoxicity ranking that is not confounded by the inter-study variability in species, strain, dosing, or assay methodology that limits comparison across the existing, largely single-agent literature.

METHODS

Study design and reagents

This is an *in vivo* experimental comparative study. It includes the use of methotrexate (50 mg/5 mL, Methotrexate EBEWE®, Ebewe Pharma, Austria); cisplatin (50 mg/50 mL, Cytoplatin-50®, Cipla, India); cyclophosphamide injection (500 mg, Cyphos®, GetWell, India); doxorubicin hydrochloride injection (10 mg, Doxoneo®, GetWell, India); foetal bovine serum (PAN-Biotech, Germany); phosphate-buffered saline, pH 7.4 (RMBIO, USA); Giemsa stain (Sigma-Aldrich, Germany); methanol (Sigma-Aldrich, Germany); glacial acetic acid (Research Products International Corp, USA); potassium chloride (Sigma-Aldrich, Germany); and colchicine (Sigma-Aldrich, Germany).

Animals and ethical considerations

Sixty male albino Swiss mice weighing 25-30 g were procured from the Animal House, College of Pharmacy, University of Baghdad. All procedures were conducted in accordance with the recommendations of the institutional Biochemical and Research Ethics Committee, following the rules of the Canadian Council on Animal Care (1984), and only after clearance by the Institutional Animal Ethics Committee. Animals were

acclimated for two days prior to the experiment under standard housing conditions (temperature $22 \pm 2^\circ\text{C}$, relative humidity 50-60%, and a 12-hour light/dark cycle). Only male mice were used in order to eliminate potential estrous-cycle-related hormonal variability as a confounding source of variance in the cytogenetic endpoints; consequently, the present findings cannot be assumed to apply equally to female animals, and possible sex-related differences in genotoxic susceptibility were not assessed and are noted as a limitation of the study.

Experimental design

Bone marrow and spleen were selected as the two target tissues for this study because both are highly proliferative and readily accessible: bone marrow is the standard, internationally validated tissue for *in vivo* cytogenetic and micronucleus testing and reflects direct effects on the primary site of hematopoiesis, whereas the spleen, as a secondary lymphoid organ that also participates in extramedullary hematopoiesis and immune surveillance, provides a complementary index of genotoxic susceptibility in a peripheral, rapidly dividing tissue; comparing the two allowed us to assess whether the relative genotoxic ranking of the four agents was consistent across a central and a peripheral proliferative compartment. Animals were randomly allocated to five groups of 12 mice each: Group 1 (negative control) received distilled water; Group 2 received a single intraperitoneal dose of cisplatin (12 mg/kg) [26]; Group 3 received a single intraperitoneal dose of methotrexate (20 mg/kg) [27]; Group 4 received a single intraperitoneal dose of doxorubicin (5 mg/kg) [28]; and Group 5 received a single intraperitoneal dose of cyclophosphamide (50 mg/kg) [29]. These single-dose regimens were selected from previously published *in vivo* genotoxicity protocols in mice [26-29] and correspond to acute, near-maximum-tolerated doses used to reliably elicit measurable cytogenetic damage within a short observation window; they are higher, on mg/kg basis, than the fractionated or cyclical doses typically used in human chemotherapy regimens and should therefore be regarded as experimental toxicological exposures intended to establish a comparative hazard ranking under standardized acute conditions, rather than as directly equivalent to clinically administered doses. Twenty-four hours after treatment, each group was randomized into two equal subgroups ($n=6$). The first subgroup was injected with colchicine (1 mg/kg, intraperitoneally) and, two hours later, was euthanized by cervical dislocation; bone marrow was aspirated from the femur under aseptic conditions for assessment of mitotic index (MI) and total chromosomal aberrations (TCA), as per a previously described protocol [30]. The second subgroup was euthanized directly by cervical dislocation, and femoral bone marrow was flushed and

processed with fetal bovine serum under aseptic conditions for analysis of micronucleus frequency [31].

Outcome measurements

The number of cells in metaphase was determined by counting 1,000 cells under light microscopy on each slide. The mitotic index (MI) was calculated as $\text{MI} = (\text{number of mitotic cells} / \text{total number of cells}) \times 100$. Bone marrow chromosome preparations for total chromosomal aberration (TCA) were carried out according to the method outlined by the working group on the *in vivo* mammalian bone marrow chromosomal aberration test [32]. Micronucleus (MN) frequency was scored in bone marrow smears as previously described [31].

Ethical considerations

The study protocol was approved by the College of Pharmacy, University of Baghdad, Iraq, RECAUBCP022678A at 17-1-2026. This study was conducted in accordance with the institutional ethical guidelines for the care and use of laboratory animals.

Statistical analysis

Data were analyzed using SPSS version 24 and are expressed as mean $\pm$ standard deviation (SD). Group comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test; $p < 0.05$ was considered statistically significant. Before performing ANOVA, the assumptions of normality and homogeneity of variance were assessed for each endpoint using the Shapiro-Wilk test and Levene's test, respectively; where these assumptions were not met, a logarithmic transformation was applied prior to analysis. All data satisfied the assumptions of normality and homogeneity of variance for ANOVA following this procedure.

RESULTS

A single intraperitoneal dose of each anticancer agent produced a significant reduction in MI relative to the distilled-water control in both bone marrow and spleen ($p < 0.05$) (Table 1).

Table 1: Mitotic index in bone marrow and spleen cells of albino mice treated with different anticancer agents compared with distilled water

Treatment Group	Mitotic Index (Bone Marrow)	Mitotic Index (Spleen)
Distilled water (negative control)	8.68 $\pm$ 0.45	7.12 $\pm$ 0.25
Methotrexate, 20 mg/kg	3.73 $\pm$ 0.25 ^a	3.24 $\pm$ 0.32 ^a
Cyclophosphamide, 50 mg/kg	2.04 $\pm$ 0.39 ^b	1.82 $\pm$ 0.46 ^b
Cisplatin, 12 mg/kg	3.18 $\pm$ 0.23 ^c	3.08 $\pm$ 0.31 ^c
Doxorubicin, 5 mg/kg	1.61 $\pm$ 0.16 ^d	1.46 $\pm$ 0.37 ^d

Data are expressed as mean $\pm$ SD; $n=6$ animals per group. ^aSignificantly different from distilled-water control ($p < 0.05$). Values with non-identical superscript letters (a, b, c, d) differ significantly from one another ($p < 0.05$).

Significant differences were also observed among the four agents in both tissues ($p < 0.05$). Doxorubicin produced the greatest suppression of MI of all agents tested, whereas methotrexate produced the smallest reduction in both bone marrow and spleen ($p < 0.05$). All four anticancer agents produced a significant increase in TCA relative to the distilled-water control in both bone marrow and spleen ($p < 0.05$) (Table 2), with significant differences also evident among the four agents ($p < 0.05$).

Table 2: Total chromosomal aberration in bone marrow and spleen cells of albino mice treated with different anticancer agents compared with distilled water

Treatment group	Total Chromosomal Aberration (Bone Marrow)	Total Chromosomal Aberration (Spleen)
Distilled water (negative control)	0.11±0.08	0.09±0.07
Methotrexate, 20 mg/kg	0.26±0.05 ^a	0.19±0.02 ^a
Cyclophosphamide, 50 mg/kg	0.46±0.03 ^{*b}	0.41±0.06 ^{*b}
Cisplatin, 12 mg/kg	0.35±0.02 ^{*c}	0.31±0.01 ^{*c}
Doxorubicin, 5 mg/kg	0.55±0.1 ^{*d}	0.51±0.11 ^{*d}

Data are expressed as mean ± SD; n=6 animals per group.

*Significantly different from distilled-water control ($p < 0.05$). Values with non-identical superscript letters (a, b, c, d) differ significantly from one another ($p < 0.05$).

Doxorubicin produced the highest frequency of TCA, while methotrexate produced the lowest, in both tissues ($p < 0.05$). Micronucleus (MN) frequency in bone marrow cells was significantly increased by each anticancer agent relative to the distilled-water control ($p < 0.05$) (Table 3), with significant differences also evident among the agents ($p < 0.05$). Doxorubicin produced the highest MN frequency and methotrexate the lowest among the agents tested ($p < 0.05$).

Table 3: Micronucleus frequency in bone marrow cells of albino mice treated with different anticancer agents compared with distilled water

Treatment group	Micronucleus Frequency (Bone Marrow)
Distilled water (negative control)	6.43±0.77
Methotrexate, 20 mg/kg	16.22±1.20 ^{*a}
Cyclophosphamide, 50 mg/kg	22.16±2.38 ^{*b}
Cisplatin, 12 mg/kg	18.17±1.39 ^{*c}
Doxorubicin, 5 mg/kg	28.43±2.23 ^{*d}

Data are expressed as mean ± SD; n=6 animals per group.

*Significantly different from distilled-water control ($p < 0.05$). Values with non-identical superscript letters (a, b, c, d) differ significantly from one another ($p < 0.05$).

DISCUSSION

Assessment of the cellular safety profile and genotoxic potential of antineoplastic drugs remains an essential part of clinical oncology and experimental toxicology. Chemotherapeutic agents are designed to impair the proliferative machinery of malignant cells; however, their mechanisms of action are not tumor-selective and can generate collateral damage in rapidly dividing normal tissues such as the bone marrow and spleen [11,12,30]. The present investigation compared the in vivo genotoxic and cytotoxic profiles of four commonly

used antineoplastic drugs—methotrexate (20 mg/kg), cyclophosphamide (50 mg/kg), cisplatin (12 mg/kg), and doxorubicin (5 mg/kg) — following a single intraperitoneal dose in albino mice, by analyzing MI, TCA, and MN frequency. These endpoints indicate the relative cytotoxicity, clastogenesis, and mutagenicity of these agents compared with a water reference. The mitotic index (MI) reflects the rate of cell proliferation and is highly sensitive to changes in cell division, making it a useful indicator of how a given agent affects proliferating tissue. In the present study, baseline MI was 8.68 in bone marrow and 7.12 in spleen cells of the control group; treatment with doxorubicin reduced these values markedly, to 1.61 and 1.46, respectively. This pronounced suppression is compatible with doxorubicin's previously reported capacity to intercalate into DNA and to stabilize the topoisomerase II-DNA cleavage complex, producing persistent strand breaks that arrest cell division and ultimately trigger cell death [24,25]. Chromosomal aberration analysis, a widely used approach for detecting DNA structural damage arising from strand breakage or faulty repair, similarly identified doxorubicin as the most genotoxic agent; this finding would be compatible with the reactive-oxygen-species-mediated DNA damage previously described for doxorubicin in other experimental systems, although ROS generation and repair-pathway activity were not directly measured in the present study, and this interpretation should therefore be regarded as inferential rather than demonstrated. Cisplatin also produced substantial chromosomal aberrations (0.35 in bone marrow and 0.31 in spleen); the formation of 1,2-intrastrand DNA cross-links that, if incompletely resolved by nucleotide excision repair, cause replication fork stalling and collapse and consequent chromosome fragmentation is a well-documented mechanism for cisplatin genotoxicity [21-23], although again this specific pathway was not verified biochemically in the present animals. Taken together, these findings are consistent with the possibility that agents that damage DNA directly—through intercalation, alkylation, or platination—are more likely than antifolate agents to induce chromosomal abnormalities within a short time frame; however, in the absence of molecular biomarkers, this remains an interpretation drawn by analogy with the published mechanistic literature rather than a mechanism established by the present data. The bone marrow micronucleus assay is an internationally validated in vivo test that captures both clastogenic (chromosome breakage) and aneugenic (spindle disruption) events; micronuclei are small extra-nuclear chromatin bodies that lag during anaphase, owing to a lack of centromeric attachment or structural fragmentation, and fail to be incorporated into daughter nuclei at telophase [8,10]. The significantly elevated MN frequency observed with cyclophosphamide (22.16 ± 2.38) is consistent with the release of acrolein and phosphoramidate mustard during hepatic

biotransformation: acrolein induces marked oxidative stress and alkylates nuclear proteins, impairing spindle apparatus integrity and generating aneugenic micronuclei containing whole chromosomes [17,19,20]. The lower, though still significant, MN frequency associated with methotrexate (16.22 ± 1.20) arises through a distinct metabolic route: as a competitive inhibitor of dihydrofolate reductase, methotrexate depletes intracellular thymidine triphosphate, leading to misincorporation of uracil into replicating DNA; subsequent base excision repair via uracil-DNA glycosylase generates persistent double-strand breaks and chromosome fragments that manifest as micronuclei [13-16]. As with the mechanistic discussion above, the acrolein/oxidative-stress and antifolate/uracil-misincorporation pathways proposed for cyclophosphamide and methotrexate, respectively, are drawn from the published literature rather than confirmed by biomarker measurements in the present animals and are offered here as plausible explanations for the observed MN frequencies rather than as mechanisms demonstrated by this study. The relative ranking observed here is broadly consistent with previously published *in vivo* rodent studies that examined these agents individually. Cyclophosphamide has repeatedly been shown to produce dose-dependent increases in bone-marrow micronucleus frequency and chromosomal breakage in mice, with the magnitude of induction in earlier reports falling within a comparable order to that observed in the present study [19,20]. Similarly, cisplatin-induced increases in chromosomal aberrations and reduced mitotic activity in murine bone marrow have been documented at doses similar to that used here [26], and doxorubicin has consistently been reported as the most potent inducer of micronucleus formation and chromosomal damage among conventional cytotoxic agents in comparable rodent models [28]. Methotrexate, by contrast, has generally produced the smallest genotoxic response among cytotoxic chemotherapeutics in prior *in vivo* work, in keeping with the present findings [27]. This concordance across independently conducted studies, using different laboratories, animal batches, and time frames, lends support to the reliability of the relative ranking reported here, although direct numerical comparison between studies is limited by differences in dosing, strain, and assay methodology.

Study Limitations

This study evaluated only a single high dose of each agent, administered once, with outcomes assessed 24 hours later; dose-response relationships and the time course of damage were not examined. The number of animals per group, while sufficient to detect large differences, may not have been adequate to detect smaller inter-agent differences. Only bone marrow and spleen were examined, so these findings should not be extrapolated to other tissues or to clinical dosing

regimens involving repeated, lower doses. Future studies should incorporate multiple dose levels, additional time points, and more specific damage biomarkers to better characterize the time course and mechanisms of genotoxicity. In particular, because only a single acute dose and a single 24-hour time point were assessed, the present design cannot distinguish whether the observed genotoxicity ranking would be maintained, attenuated, or reversed at lower, clinically fractionated doses; at later time points once DNA-repair processes have had time to act; or after repeated dosing as used clinically; it is therefore possible that the relative ordering of agents reported here is specific to this acute, high-dose, 24-hour model and does not directly predict genotoxic risk under chronic clinical dosing. In addition, only male mice were studied, so sex-related differences in drug metabolism and genotoxic susceptibility could not be evaluated, and no molecular or mechanistic biomarkers (e.g., markers of oxidative stress or DNA-repair pathway activity) were measured, so the mechanistic interpretations offered in the Discussion remain inferences drawn from the existing literature rather than findings directly demonstrated by this study.

Conclusion

A consistent inverse relationship between structural genomic damage and cell proliferation was observed across the four antineoplastic agents tested, with doxorubicin at the top of this toxicological hierarchy, followed by cyclophosphamide, cisplatin, and methotrexate, under the single-dose, 24-hour, male-mouse experimental conditions examined here. This hierarchy is qualitatively consistent with the principal dose-limiting adverse effects reported in human oncology, such as myelosuppression, leukopenia, aplastic anemia, and secondary immunodeficiency; however, this parallel is qualitative, and the present ranking should be interpreted strictly within the conditions of this acute experimental model. Given the single acute dose, single time point, absence of mechanistic biomarkers, and use of male animals only, this genotoxicity ranking should not be generalized to overall clinical toxicity, to female patients, or to the fractionated, repeated-dose regimens used therapeutically, and should instead be regarded as a hypothesis-generating comparison to be confirmed by studies incorporating a wider dose range, multiple time points, both sexes, and direct mechanistic biomarkers.

Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

The data that supports the findings of this study are available from the corresponding author upon a reasonable request

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