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(*As of February 2026*)

# CONTENTS

*Volume 20, Number 3, 2026*

## Policy Forum

**205-210** **Are we ready to replace animal models? A perspective on regulatory challenges of human-relevant drug testing systems**
*Tanbin Liu, Hongzhou Lu*

## Original Article

**211-220** **Low rate of pharmacists accessing renal function laboratory values: A cross-sectional study using electronic medical records of a Japanese community pharmacy**
*Shingo Kondo, Nozomi Ito, Mari Maese, Yuko Okamoto, Hiroki Iwata, Noriko Kobayashi, Katsunori Yamaura*

**221-227** **Metabolic and endocrinological effects of weekly growth hormone replacement therapy with somapacitan in patients with adult growth hormone deficiency after switching from daily growth hormone replacement therapy: A real-world exploratory cohort study**
*Ichiro Abe, Keita Kubo, Yuya Fujita, Kentaro Ochi, Toshiaki Wada, Ryo Mitsuoka, Kaori Takeshita, Ayuko Higashi, Tamotsu Kato, Tadachika Kudo, Hideaki Shimada, Osamu Imakyure, Makiko Abe, Shigeaki Mukoubara, Kunihisa Kobayashi*

**228-234** **Association of epidural analgesia with childbirth satisfaction among women with severe fear of childbirth: A cross-sectional study**
*Ayako Hayashi, Akari Kanesaki, Nobuhiko Suganuma*

**235-244** **Seasonality of psychiatric symptoms in older adults in long-term psychiatric care**
*Tomoki Kakehashi, Yuma Sonoda*

**245-253** **Exploratory study on the effect of ferulic acid derived from rice bran on dull skin**
*Jingxiu Zhou, Mizuka Kobayashi, Hanaka Kimura, Yuki Inoue, Tomoki Oguro, Toshio Nakamura, Takuo Tsuno, Kanae Mure, Masatoshi Jinnin, Yasushi Shigeri*

**254-263** **Lactoferrin improves scopolamine-induced memory impairment in mice**
*Noa Mizukami, Masayuki Shibuya, Megumi Furukawa, Ryoken Aoki, Kanata Murakami, Masahiro Toho, Daichi Nagashima, Nobuo Izumo*

## Brief Report

**264-268** **Docosahexaenoic acid increases tyrosine hydroxylase phosphorylation at Ser40 without increasing tyrosine hydroxylase protein expression in differentiated NG108-15 cells**
*Daisuke Miyazawa, Natsumi Katsurayama, Hikari Sato, Kinari Suzuki, Tomoko Tahira, Hideki Mizutani*

**264-268** **Analytical method validation and feasibility of salivary pregabalin measurement in Japanese volunteers: A pilot study**
*Hina Itoi, Yoshito Gando, Takeo Yasu, Masayuki Kobayashi*

273-279 **Associations between wound exudate amino acid profiles and microbial dissimilarity in wound and peri-wound skin in healing wounds**
*Mao Kunimitsu, Makoto Tsunoda, Tomoko Akase, Mikio Kinoshita, Gojiro Nakagami*

***Policy Forum***

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# Are we ready to replace animal models? A perspective on regulatory challenges of human-relevant drug testing systems

**Tanbin Liu, Hongzhou Lu***

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**SUMMARY**: This perspective examines whether animal models can be gradually replaced by human-relevant New Approach Methodologies (NAMs) in drug development and regulatory evaluation, synthesizing recent advances across hepatology, oncology, neurotoxicology, and cardiac safety assessment. Although the relevant technologies have become increasingly mature, their acceptance at the regulatory level is still lagging due to factors such as limited standardization, insufficient reproducibility of results, and unclear approval pathways. Building on a systematic comparison of existing evidence, this article proposes a three-stage transition pathway: the first stage (auxiliary stage)—using NAMs in parallel with animal experiments; the second stage (partial replacement stage)—using NAMs as the preferred efficacy evaluation model for human-specific diseases; and the third stage (regulatory integration stage). Only through gradual validation can animal models be replaced responsibly, thereby increasing the success rate of translational research and reducing the use of animal experiments. Overcoming the persistent challenges of limited standardization, poor reproducibility, and unclear regulatory pathways is the central prerequisite for this transition.

***Keywords***: New Approach Methodologies (NAMs), regulatory acceptance, three stage roadmap, translational research

## 1. Introduction

For decades, animal models have served as the gold standard for preclinical drug evaluation. However, they have consistently demonstrated limited capacity to predict human therapeutic responses. This limitation is particularly pronounced in areas such as infectious diseases, immunology, and hepatology. A salient illustration is oncology, where overall clinical trial failure rates exceed 90%—a figure that, in part, reflects the inadequate translational validity of conventional animal-based preclinical systems (*1-3*). In parallel, mounting scientific efforts and increasingly rigorous ethical standards have catalyzed rapid advances in human-relevant *in vitro* research platforms, encompassing organ-on-a-chip technologies, induced pluripotent stem cell (iPSC)-derived models, and computational toxicology approaches (*4,5*). The formal recognition of New Approach Methodologies (NAMs) by the U.S. Food and Drug Administration (FDA) marks a significant inflection point in this trajectory, signaling a broader paradigm shift in regulatory thinking (*6,7*). Despite this momentum, a critical bottleneck persists: the transition from technological feasibility to regulatory acceptance remains incomplete. In the absence of clear, internationally harmonized validation and qualification frameworks, NAMs risk being relegated to exploratory research contexts rather than being adopted as recognized standards for regulatory submissions. Bridging this regulatory gap is therefore essential to realizing the full translational potential of these advanced methodologies.

Unlike prior reviews that focused primarily on technological advances in organoids or organ-on-a-chip platforms, the present article specifically focuses on the transition pathway from technological maturity to regulatory acceptance. We propose a three-stage model stratified by disease type and the maturity of NAM platforms and emphasize a fit for purpose validation framework rather than a binary argument for or against complete animal model replacement. This perspective is intended to bridge the gap between scientific innovation and regulatory practice.

## 2. Why animal models fall short: A clinical perspective

From the perspective of clinical researchers, the gap between animal trial outcomes and human clinical

effectiveness is striking. In the realm of liver disorders, numerous potential NASH medications that demonstrated effectiveness in mouse models were found to be useless in human clinical trials (*8,9*); similarly, hepatitis B virus (HBV)—owing to its highly restricted species tropism, rendering it able to efficiently infect only humans and higher primates—lacks acceptable animal models, markedly hampering preclinical antiviral testing (*10*). In the field of neurodegenerative disease research, dozens of potential drugs for Alzheimer's disease that alleviated amyloid pathology in transgenic mice ultimately failed to result in cognitive improvement in human patients (*11-13*). In oncology, mouse xenograft models typically fail to correctly represent the human tumor microenvironment and immune evasion mechanisms, explaining why many immunotherapies that are effective in mice have little impact on people (*14-16*). Even in the study of infectious diseases and sepsis, rodent models fail to replicate the unique cytokine responses and Toll-like receptor signaling pathways in humans, leading to repeated setbacks in clinical translation (*17-19*). This situation is not uncommon in biomedical research, and a well known adage succinctly captures this persistent disconnect: "The drug candidate demonstrated marked preclinical efficacy but failed to be clinically useful."

These failures share a common mechanistic basis. First, there are significant differences in the immune system: the evolutionary pathways of the innate and adaptive immune systems in mice and humans are vastly different; mice's responses to viral infections or checkpoint inhibitors often differ greatly from actual responses in patients (*20,21*). Secondly, the viral life cycle is species-specific: HBV, HCV, and many emerging viruses cannot replicate in conventional laboratory animals without extensive genetic modification. Third, there are significant differences in drug metabolism kinetics and toxicity characteristics: a drug that is metabolized safely in the rat liver may produce toxic metabolites in human liver cells, and *vice versa*. These differences are not merely theoretical; they have directly led to several catastrophic drug recall events, such as the cytokine storm caused by TGN1412: this compound appeared safe in monkey experiments but nearly resulted in the death of human volunteers (*22*).

## 3. The emerging approach: Human-relevant efficacy modeling

In recent years, remarkable developments in different therapeutic domains have revealed that human-related biological systems are no longer simple conceptual assumptions but have become mature platforms confirmed by functional testing. In the area of hepatology, a recent study effectively replicated the whole life cycle of the hepatitis B virus (HBV) from invasion to antiviral response using liver organoids grown from iPSCs (*23*). In the field of immuno-oncology, microphysiological systems constructed based on patient-derived organoids, perfusable vascular networks, and tumor-associated macrophages have confirmed the macrophage-mediated immunosuppressive effect of immunotherapies in various solid tumor models, including prostate cancer and hepatocellular carcinoma (*24-26*). In cardiac toxicity research, a beating heart-on-a-chip platform based on human iPSC-derived cardiomyocytes achieved an accuracy of 91.6% in detecting drug-induced QT interval prolongation (*27*). In the realm of neurotoxicology, brain organoids containing iPSC-derived microglia efficiently reproduced the microglia-mediated inflammatory response to developing neurotoxins, a route commonly missed in normal animal research (*28*).

These examples highlight a shift in validation logic: from "Does it behave like a sick animal?" to "Can it predict clinical outcomes in humans?" However, not all NAM platforms are equally mature. We propose a simple two-tier stratification:

**Tier 1 (relatively mature)**: iPSC-cardiomyocyte models for cardiotoxicity (CiPA paradigm, > 90% retrospective accuracy) and liver organoids/chips for DILI efficacy. These have received positive regulatory signals in defined contexts of use.

**Tier 2 (exploratory)**: Multi-organ systems, neuroimmune models, and vascularized tumor chips. These suffer from low throughput, high batch-to-batch variability, and lack of cross-laboratory validation. They remain research tools and are not regulatory-ready.

## 4. The real problem: Technological readiness does not equal regulatory readiness

However, human-relevant platforms cannot fully replace animal models in the short term. An objective assessment must acknowledge several crucial shortcomings. Even for Tier 1 platforms, organoid and organ on a chip technologies vary significantly across laboratories (*e.g.*, extracellular matrices, culture media, differentiation protocols, and endpoints), making direct comparison of results difficult. Second, the level of batch-to-batch variability is high: even within the same laboratory, iPSC derived organoids often lack consistency in size, cellular composition, and functional maturity. Third, there is a lack of long-term toxicity and systemic response data. Most current platforms operate for only a few days to weeks and cannot integrate the neuroendocrine-immune axis, limiting their ability to assess adverse reactions such as delayed cardiotoxicity, cytokine release syndrome, and tissue remodeling. Standard liver chips typically support culture for only 21–28 days, insufficient to capture drug induced liver injury (DILI) that may manifest after months of treatment. Similarly, kidney on-a-chip models are largely restricted to acute injury endpoints, with few capable of recreating progressive fibrosis or chronic tubular atrophy. These gaps represent major hurdles for regulatory acceptance, as many drug withdrawals are

due to late onset toxicities not detectable in short term assays. Finally, regulatory certification pathways remain unclear. Although the U.S. FDA's NAM program is a positive signal, no global consensus exists on what validation data suffice to replace animal testing for specific indications. Thus, technological maturity does not equal regulatory maturity. Without coordinated efforts to standardize production processes, ensure the reproducibility of benchmark results, and establish fit for purpose certification frameworks, human based systems risk becoming scientific curiosities rather than regulatory tools. Acknowledging these shortcomings does not negate the value of NAMs but rather points the way toward the next critical improvements. For Tier 2 platforms, these challenges are even more pronounced.

## 5. Current regulatory landscape of NAMs

A systematic comparison of major regulatory bodies reveals significant heterogeneity in NAM acceptance. The U.S. FDA has taken the most proactive stance through its NAM program and ISTAND initiative, issuing draft guidance on liver and cardiac safety models and accepting organoid data for certain IND submissions (*e.g.*, rare disease gene therapies). The European Medicines Agency (EMA) has incorporated NAMs into its 3Rs strategy and published a reflection paper on organ-on-chip technology, but it requires parallel animal data for most indications. Japan's Pharmaceuticals and Medical Devices Agency (PMDA) has supported the development of iPSC-based cardiotoxicity assays (CiPA) and accepts them as supplemental evidence, and yet it retains animal testing as the default standard. China's National Medical Products Administration (NMPA) has recently issued guidance on organoid research but has not formally recognized any NAM platform for regulatory submission; ongoing pilot projects focus on liver toxicity screening. The International Council for Harmonisation (ICH) has not issued unified NAM guidelines, though ICH S5 (reproductive toxicology) and ICH M3 (nonclinical safety) are undergoing review for potential updates. This fragmented landscape underscores the urgent need for global coordination.

## 6. A path forward: The three phase transition model

We are not yet ready for a complete replacement—but the transition is both necessary and achievable. This paper therefore proposes a three-phase roadmap for the responsible integration of NAMs into regulatory practice as shown in Figure 1. Given that technological maturity and regulatory readiness vary across disease contexts and platform types, the phases need not advance simultaneously across all therapeutic areas; the timelines below (~2028, 2028-2033, 2033 and beyond) are illustrative projections intended to guide planning and

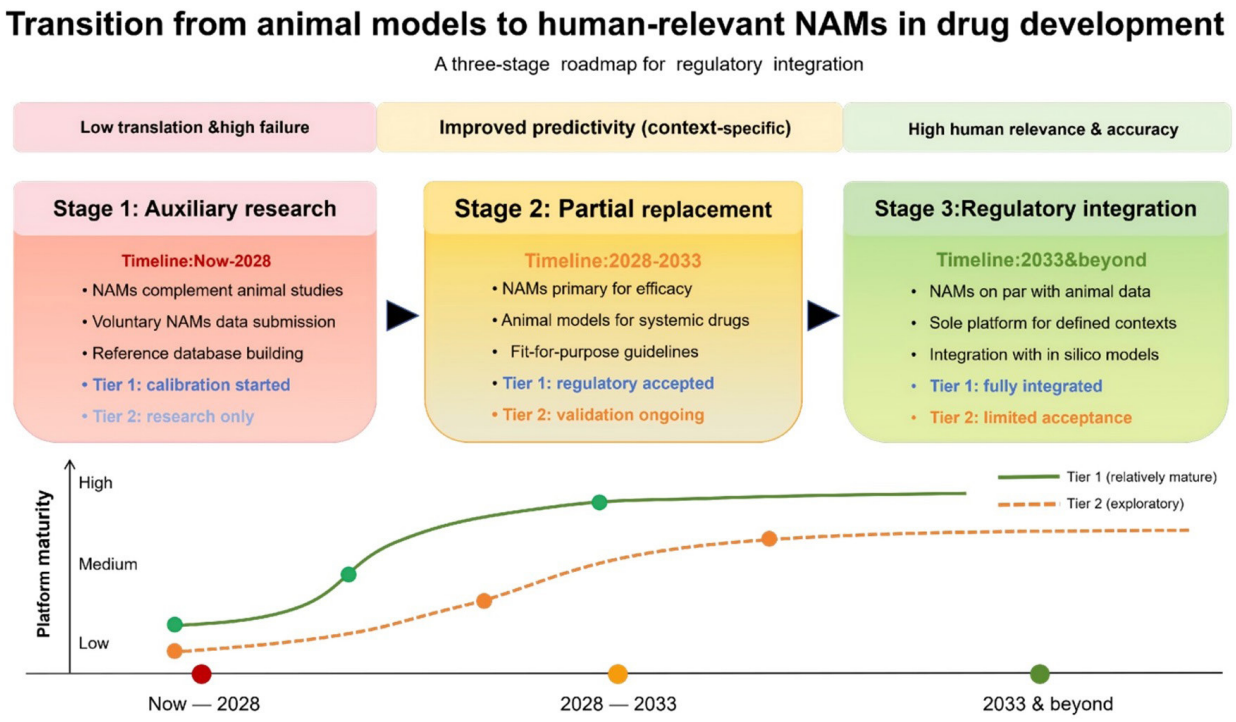


**Figure 1. Proposed three phase roadmap for integrating NAMs into regulatory drug development.** Stage 1 (auxiliary, ~2028): NAMs used in parallel with animal studies; voluntary data submission; reference databases established; Tier 1 platforms begin calibration. Stage 2 (partial replacement, 2028–2033): NAMs become the primary efficacy model; fit-for-purpose guidelines issued; Tier 1 platforms accepted, Tier 2 platforms undergo validation. Stage 3 (full integration, 2033+): NAM data on par with animal data for IND submissions; sole platform for defined contexts; integrated with in silico models; ICH guidelines harmonized. Timelines are illustrative; Tier 1 (relatively mature) platforms progress faster than Tier 2 (exploratory) platforms.

are not fixed mandates.

**Stage 1: Auxiliary research (near term, illustratively by ~2028)**

In this phase, NAMs function as complementary tools to conventional animal research. For each candidate drug, sponsors need to concurrently conduct conventional animal efficacy and toxicology studies as well as a succession of human-relevant NAM assessments—such as using iPSC-derived liver organoids or liver chips to demonstrate effectiveness and early toxicity analysis for liver-targeted therapies. Regulatory bodies do not yet accept NAM data alone, but they will begin to develop reference databases to study the association between organoid responses and human efficacy. This phase involves extremely minimal regulatory revisions, including a proposal for sponsors to voluntarily provide NAM data. The fundamental purpose is to acquire real-world evidence: When organoid models predict effectiveness, do they have a greater ability to predict the outcomes of Phase II clinical trials compared to animal experiments? During this stage, efforts should also begin to establish reference standards and interlaboratory calibration protocols for highly mature NAMs (*e.g.*, liver chips and iPSC cardiomyocytes), whereas less mature platforms (*e.g.*, multi organ systems and neuroimmune models) would remain primarily research tools.

**Stage 2: Partial replacement (mid term, illustratively 2028–2033)**

By the late 2020s, specific therapeutic areas can, after thorough validation, gradually transition to a partial replacement model. For human-specific pathogens such as HBV and human immunodeficiency virus (HIV) as well as newly emerging viruses that lack natural animal hosts, NAMs should become the primary efficacy evaluation model. In contrast, for immunotherapies or drugs requiring complex systemic pharmacokinetic assessment, animal models remain essential in Stage 2, with NAMs serving in a supportive role. Similarly, in drug screening, for certain categories of compounds (such as direct-acting antiviral drugs and low-molecular-weight compounds with simple metabolic pathways), patient-derived organoid panels with diverse genetic backgrounds could potentially reduce or replace the use of dogs and rats, pending systematic validation and regulatory qualification. Regulatory agencies will issue clear guidelines: for example, "For HBV antiviral drugs, new drug clinical trial (IND) applications can rely on efficacy data obtained from organoids; efficacy evaluation does not require the use of animal models and is only needed to assess safety endpoints that NAMs cannot yet cover". During this stage, we encourage regulatory agencies to define fit-for-purpose validation criteria for specific NAM platforms, recognizing that not all NAMs are equally mature.

**Stage 3: Integration into the regulatory framework (long term, illustratively 2033 and beyond)**

In the final stage, NAMs are expected to become a standard and widely recognized core component of new drug clinical trial (IND) submission materials. Organoid and organ-on-a-chip data will be on par with animal experiment data, with both serving as sources of evidence, each with its unique advantages and limitations. For certain clearly defined situations (such as drugs metabolized by the liver or viruses that directly cause cell damage), NAMs can even serve as the sole platform for evaluating efficacy and safety, thereby completely replacing animal experiments. In parallel, in silico models—such as quantitative systems pharmacology (QSP), physiologically based pharmacokinetic (PBPK) modeling, and machine learning-based toxicity predictors—are expected to be integrated with organoid and organ on-a-chip data, collectively comprising a comprehensive NAM toolbox for regulatory decision making. Achieving this goal not only requires continued maturation of the technology but also necessitates the revision of relevant ICH guidelines and the promotion of global coordination among the NMPA, FDA, EMA, and PMDA. Although the transformation process may be relatively slow, as long as we start now to systematically build an evidence base, this goal is entirely achievable.

Figure 1 shows the three stage transition model. The timelines (~2028, 2028–2033, 2033 and beyond) are illustrative and assume differential progression across Tier 1 and 2 platforms. Tier 1 platforms (*e.g.*, liver chips and iPSC cardiomyocytes) are expected to enter Stage 2 earlier than Tier 2 platforms (*e.g.*, multi organ systems).

**7. Limitations of this perspective**

Our proposed roadmap is intentionally optimistic. It may underestimate the lasting need for animal models of complex systemic diseases (*e.g.*, autoimmune disorders and chronic neuroinflammation) where human-relevant platforms are still immature. Global regulatory harmonization remains a formidable political and logistical challenge. This perspective is meant to stimulate discussion, not to serve as a regulatory template.

**8. Conclusion**

"Are we ready to replace animal models?" This question seems to elicit a binary answer, but reality indicates that the answer lies along a gradient. From a technical perspective, human-relevant systems such as liver organoids have, in defined contexts of use, demonstrated performance that can surpass animal models. Research on the HBV liver organoid model is just one of many

signs that in the future we will no longer rely on mice or monkeys to replace human patients. However, the regulatory system that was meticulously built over half a century based on animal data has yet to keep pace with this change. As mentioned at the beginning, the real bottleneck is increasingly shifting from the capacity for innovation to acceptance by the regulatory system.

We cannot simply ask regulatory agencies to abandon their cautious approach—their mission is to protect public health and to ensure incidents like TGN1412 do not happen again. But by adopting a three-stage model, we can accelerate this transition process: first as an auxiliary means, then achieving partial replacement, and finally achieving full regulatory integration. The path is clear, and the tools are in place. What we need now is a shared commitment from researchers, sponsors, and regulators to move forward with caution and avoid unnecessary delays. Achieving this vision requires not only continued technological refinement but also harmonized regulatory frameworks across the FDA, EMA, PMDA, NMPA, and ICH, as outlined in Section 5.

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**References**

1. Maeda H, Khatami M. Analyses of repeated failures in cancer therapy for solid tumors: Poor tumor-selective drug delivery, low therapeutic efficacy and unsustainable costs. Clin Transl Med. 2018; 7:11.
2. Van Norman GA. Limitations of animal studies for predicting toxicity in clinical trials: Is it time to rethink our current approach? JACC Basic Transl Sci. 2019; 4:845-854.
3. Mak IW, Evaniew N, Ghert M. Lost in translation: Animal models and clinical trials in cancer treatment. Am J Transl Res. 2014; 6:114-118.
4. Ingber DE. Human organs-on-chips for disease modelling, drug development and personalized medicine. Nat Rev Genet. 2022; 23:467-491.
5. Youhanna S, Kemas AM, Preiss L, Zhou Y, Shen JX, Cakal SD, Paqualini FS, Goparaju SK, Shafagh RZ, Lind JU, Sellgren CM, Lauschke VM. Organotypic and microphysiological human tissue models for drug discovery and development-Current state-of-the-art and future perspectives. Pharmacol Rev. 2022; 74:141-206.
6. Avila AM, Bebenek I, Bonzo JA, *et al*. An FDA/CDER perspective on nonclinical testing strategies: Classical toxicology approaches and new approach methodologies (NAMs). Regul Toxicol Pharmacol. 2020; 114:104662.
7. Yao J, Peretz J, Bebenek I, Avila A, Alapatt T, Lee B, Patel D, Brown P, Davis-Bruno K. FDA/CDER/OND experience with New Approach Methodologies (NAMs). Int J Toxicol. 2026; 45:136-156.
8. Hunter H, de Gracia Hahn D, Duret A, *et al*. Weight loss, insulin resistance, and study design confound results in a meta-analysis of animal models of fatty liver. Elife. 2020; 9:e56573.
9. Gallage S, Avila JEB, Ramadori P, Focaccia E, Rahbari M, Ali A, Malek NP, Anstee QM, Heikenwalder M. A researcher's guide to preclinical mouse NASH models. Nat Metab. 2022; 4:1632-1649.
10. Liu Y, Maya S, Ploss A. Animal models of hepatitis B virus infection-Success, challenges, and future directions. Viruses. 2021; 13:777.
11. Granzotto A, Vissel B, Sensi SL. Lost in translation: Inconvenient truths on the utility of mouse models in Alzheimer's disease research. Elife. 2024; 13:e90633.
12. Weekman EM, Sudduth TL, Caverly CN, Kopper TJ, Phillips OW, Powell DK, Wilcock DM. Reduced efficacy of anti-Abeta immunotherapy in a mouse model of amyloid deposition and vascular cognitive impairment comorbidity. J Neurosci. 2016; 36:9896-9907.
13. Kodamullil AT, Iyappan A, Karki R, Madan S, Younesi E, Hofmann-Apitius M. Of Mice and men: Comparative analysis of neuro-inflammatory mechanisms in human and mouse using cause-and-effect models. J Alzheimers Dis. 2017; 59:1045-1055.
14. Lonberg N. The Problem with syngeneic mouse tumor models. Cancer Immunol Res. 2025; 13:456-462.
15. Floe LVB, Pedersen MG, Moller BK. Animal models in preclinical evaluation of CAR-T cell therapy: Advantages and limitations. Biochim Biophys Acta Rev Cancer. 2025; 1880:189455.
16. Hendriks H. Advancing oncology drug development: Innovative approaches to enhance success rates while reducing animal testing. Biochim Biophys Acta Rev Cancer. 2025; 1880:189467.
17. Seok J, Warren HS, Cuenca AG, *et al*. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A. 2013; 110:3507-3512.
18. Stortz JA, Raymond SL, Mira JC, Moldawer LL, Mohr AM, Efron PA. Murine models of sepsis and trauma: Can we bridge the gap? ILAR J. 2017; 58:90-105.
19. Jameson SC, Masopust D. What is the predictive value of animal models for vaccine efficacy in humans? Reevaluating the potential of mouse models for the human immune system. Cold Spring Harb Perspect Biol. 2018; 10:a029132.
20. Masubuchi T, Chen L, Marcel N, *et al*. Functional differences between rodent and human PD-1 linked to evolutionary divergence. Sci Immunol. 2025; 10:eads6295.
21. Zschaler J, Schlorke D, Arnhold J. Differences in innate immune response between man and mouse. Crit Rev Immunol. 2014; 34:433-454.
22. Attarwala H. TGN1412: From discovery to disaster. J Young Pharm. 2010; 2:332-336.
23. Liu T, Xu J, Chen X, Li J, Ke J, Xu J, Lu H, Wu F. Modeling of the hepatitis B virus life cycle and the efficacy of antivirals in human iPSC-derived hepatic

organoids. Biosci Trends. 2026; 20:235-244.
24. Bains RS, Raju TG, Semaan LC, Block A, Yamaguchi Y, Priceman SJ, George SC, Shirure VS. Vascularized tumor-on-a-chip to investigate immunosuppression of CAR-T cells. Lab Chip. 2025; 25:2390-2400.
25. Zou Z, Lin Z, Wu C, Tan J, Zhang J, Peng Y, Zhang K, Li J, Wu M, Zhang Y. Micro-engineered organoid-on-a-chip based on mesenchymal stromal cells to predict immunotherapy responses of HCC patients. Adv Sci (Weinh). 2023; 10:e2302640.
26. Sanchez-de-Diego C, Yada RC, Sethakorn N, *et al*. Engineering the bone metastatic prostate cancer niche through a microphysiological system to report patient-specific treatment response. Commun Biol. 2025; 8:961.
27. Visone R, Lozano-Juan F, Marzorati S, Rivolta MW, Pesenti E, Redaelli A, Sassi R, Rasponi M, Occhetta P. Predicting human cardiac QT alterations and pro-arrhythmic effects of compounds with a 3D beating heart-on-chip platform. Toxicol Sci. 2023; 191:47-60.
28. Yuan NY, Richards WD, Parham KT, Clark SG, Greuel K, Polzin B, Smith SW, Lebakken CS. Neural organoids incorporating microglia to assess neuroinflammation and toxicities induced by known developmental neurotoxins. Curr Res Toxicol. 2025; 9:100252.

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# Low rate of pharmacists accessing renal function laboratory values: A cross-sectional study using electronic medical records of a Japanese community pharmacy

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**SUMMARY**: This study aimed to assess the availability of renal function laboratory values in Japanese community pharmacies and examine their association with the prescription of drugs requiring dosage adjustment. In this cross-sectional observational study, data were obtained from the electronic medical records of a community pharmacy in Japan. A total of 389 eligible patients (aged ≥ 18 years) who visited the pharmacy in April 2024 were included. The primary outcome measures were the proportion of participants with available renal function laboratory values and those prescribed medications requiring dosage adjustment. A total of 128 patients (32.9%) were prescribed at least one medication requiring dosage adjustment, most commonly vitamin A and D preparations (32 cases) and antidiabetic agents (31 cases). Renal function laboratory values were available for 40 patients (10.3%). Compared to those not prescribed such drugs, patients receiving medications requiring renal-based dosage adjustments included a significantly higher proportion of older adults (70/128, 54.7% *vs* 98/261, 37.5%, $p = 0.001$) and patients with available laboratory values (20/128, 15.6% *vs* 20/261, 7.7%, $p = 0.015$). No patients were identified with the "Triple Whammy," which is known to significantly increase the risk of renal function deterioration. Community pharmacists endeavor to obtain renal function laboratory values for older adults, who are more likely to be prescribed medications requiring dosage adjustment. However, the proportion of cases with obtained laboratory values remains low. Pharmacists are expected to actively recommend renal function testing to physicians. Furthermore, they should contribute to safe pharmacotherapy by utilizing comprehensive patient information.

***Keywords***: community pharmacy services, renal insufficiency, patient safety, aged

## 1. Introduction

The kidneys play a pivotal role in maintaining fluid homeostasis and excreting waste products. Chronic kidney disease (CKD) is defined as a condition characterized by structural or functional abnormalities of the kidney—such as glomerular filtration rate (GFR) < 60 mL/min/1.73 $m^2$ or albuminuria ≥ 30 mg per 24 hours—persisting for more than three months (*1*). The global prevalence of CKD is estimated at approximately 13.4% and continues to rise (*2,3*). In Japan, approximately 20% of adults (about 19.9 million people) are estimated to have stages 3–5 CKD (*4*). Patients with CKD represent a population with significant opportunities for therapeutic intervention, as they frequently have comorbidities such as diabetes, hypertension, and dyslipidemia. Moreover, because most patients with CKD have mild to moderate disease (stages 1–3), they can typically be managed by general practitioners in outpatient rather than inpatient settings (*2*). As of October 1, 2023, there were 36.23 million people aged 65 years or older in Japan, accounting for 29.1% of the total population. Renal function declines with age, highlighting the importance of considering age-related changes in healthy older individuals (*5*). With Japan's rapidly aging population, the number of patients with impaired renal function is expected to increase. In addition to estimated GFR (eGFR), estimated creatinine clearance (eCCr) based on serum creatinine (Cr) is commonly used to assess renal function in clinical practice. The eCCr, calculated using the Cockcroft-Gault formula based on serum Cr levels, age, gender, and weight, is commonly used to determine drug dosage. Community pharmacies are in a key position to prevent adverse events, as they frequently fill prescriptions for patients with impaired

renal function. However, community pharmacies often lack access to laboratory values related to patients' renal function (*6*). In Japan, a system utilizing the "My Number Card" has allowed healthcare professionals, including community pharmacists, to access annual health checkup results since October 2021. However, updates are often delayed, and available data typically date back about a year.

A decline in renal function leads to elevated blood concentrations of renally excreted drugs, thereby increasing the likelihood of adverse drug reactions (*7*). Many drugs require dosage adjustment in patients with impaired renal function (*8*). Community pharmacists are not sufficiently involved in dosage adjustment based on renal function (*9*). Nevertheless, appropriate dosage adjustment is essential to prevent adverse events caused by overdose in patients with renal impairment. Therefore, in Japan, community pharmacies commonly refer to the 37th edition of the Dosage Recommendations for Drugs That Require the Most Attention in Renal Impairment, published by the Japanese Society of Nephrology and Pharmacotherapy (JSNP) (*10*). This list is freely accessible and widely used in hospitals and pharmacies, as it is distributed at no cost in accordance with the intentions of the academic society.

A major cause of drug-induced kidney injury is the use of glycopeptide antibiotics or non-steroidal anti-inflammatory drugs (NSAIDs) (*11*). Specifically, a rapid decline in renal function occurring within a few hours to days is commonly referred to as acute kidney injury (AKI). Approximately a decade ago, a study reported that the concurrent use of three specific drug classes—renin-angiotensin system (RAS) inhibitors, which include angiotensin-converting enzyme inhibitors (ACE-Is) and/or angiotensin receptor blockers (ARBs), diuretics, and NSAIDs—increases the risk of AKI, a phenomenon known as the 'Triple Whammy' (*12*). Since these drugs are all commonly prescribed, monitoring this combination is crucial. Community pharmacists, who can manage medications prescribed by multiple clinics, play an essential role in this process by applying their expertise.

When community pharmacies adjust dosages based on renal function, laboratory values are essential for appropriate decision making. Community pharmacists must identify drugs requiring dosage adjustment and determine which patients have been prescribed them. However, prescription patterns requiring renal-based dosage adjustments and the availability of relevant laboratory values remain unclear in clinical practice. This study aims to quantify drugs requiring renal-based dosage adjustment and identify the number of patients prescribed them at the Keio University Community Pharmacy. This single-center, retrospective cross-sectional study was conducted not to provide a national estimate, but to describe the profile of high-risk drugs, the sources of laboratory data, and prescription combination patterns in a real-world community pharmacy. The findings of this study will enhance pharmacists' awareness of renal dose adjustment and support the provision of appropriate drug therapy.

## 2. Materials and Methods

### 2.1. Study design

We conducted a cross-sectional study to investigate how renal function laboratory values are utilized in pharmacies, referencing a large volume of patient medication records from a single community pharmacy. Patient age, sex, availability of renal function laboratory values, their sources, and prescribed medications were extracted and entered into an Excel spreadsheet for analysis. Renal function laboratory values in this study were assessed using eGFR, Cr, and blood urea nitrogen (BUN). A list of drugs requiring renal-based dosage adjustment was created to analyze the prescribed medications. From the pharmacy's drug list, medications included in the 'Dosage Recommendations for Drugs That Require the Most Attention in Renal Impairment, 37th Edition' were selected. This reference, published by the JSNP, was compiled using data from prescription drug package inserts.

### 2.2. Study population

Information about the study was disclosed within the pharmacy, and patients were given the opportunity to decline participation through an opt-out procedure. Medication histories of patients who visited the Keio University Community Pharmacy with prescriptions between April 1 and April 25, 2024, were included. Patients under 18 years were excluded due to the use of a different renal function estimation formula in this age group. Homebound patients were not included in the study population because there was no direct interaction with them at the pharmacy. Furthermore, patients receiving only topical prescriptions were not included in the study population, because these medications do not require renal-based dosage adjustment.

### 2.3. Statistical analysis

Drug classification was performed using the system published by the Ministry of Health, Labour and Welfare (MHLW), and the number of drugs per therapeutic category was visualized through simple tabulation. Renal function data were considered available if at least one of eGFR, BUN, or Cr was obtainable. For the analysis of age-related differences, patients were categorized into "older adults" (aged ≥ 65 years) and "non-older adults" (aged 18–64 years). The availability of renal function data and the proportion of older adults were analyzed using Pearson's chi-squared test in Microsoft Excel.

The normality of eGFR values was assessed using the Shapiro–Wilk test, and the equality of variances was assessed using the F-test in RStudio. Based on these assessments, eGFR values were compared between older and non-older adults using a two-sided Student's *t*-test. Box plots were generated using RStudio software (version 2026.01.1+403; Posit Software, PBC, Boston, MA, USA). A $p$-value < 0.05 was considered statistically significant. The number of patients prescribed each drug class involved in the Triple Whammy was illustrated using a Venn diagram.

2.4. Ethics approval

The study protocol was submitted to and approved by the Ethics Committee of the Faculty of Pharmacy, Keio University (approval No. 241113-1). Information about the study was disclosed within the pharmacy, and individuals were given the opportunity to decline participation through an opt-out procedure.

## 3. Results

3.1. Adopted medications requiring renal dose adjustment in the pharmacy

This study aimed to calculate the proportion of renal function laboratory values recorded in medication histories at a community pharmacy. Prior to initiating the study, a list of pharmacy-adopted medications requiring dose adjustments based on renal function was created using a drug list published by the JSNP (Figure 1). The medications were categorized according to their therapeutic classification. The Keio University Community Pharmacy adopted 1,521 pharmaceutical products, including different dosage forms of the same active ingredients. From these, only oral medications and liquid preparations were extracted, resulting in 1,239 products. When grouped by ingredients, the total decreased to 697, of which 57 ingredients (8.2%) matched those on the JSNP list. The Keio University Community Pharmacy carried 148 products containing the 57 identified ingredients, accounting for 12% of the 1,239 listed products.

The drugs were categorized into 23 therapeutic classes (Figure 2). Among them, the most common were 26 antidiabetic agents, 19 central nervous system agents, 16 vitamin A and D preparations, and 10 anticoagulants. Diabetic nephropathy develops because of vascular damage caused by hyperglycemia in diabetes mellitus. Therefore, patients with these conditions require closer monitoring.

3.2. Characteristics of patients included in this study

Patients who visited the Keio University Community

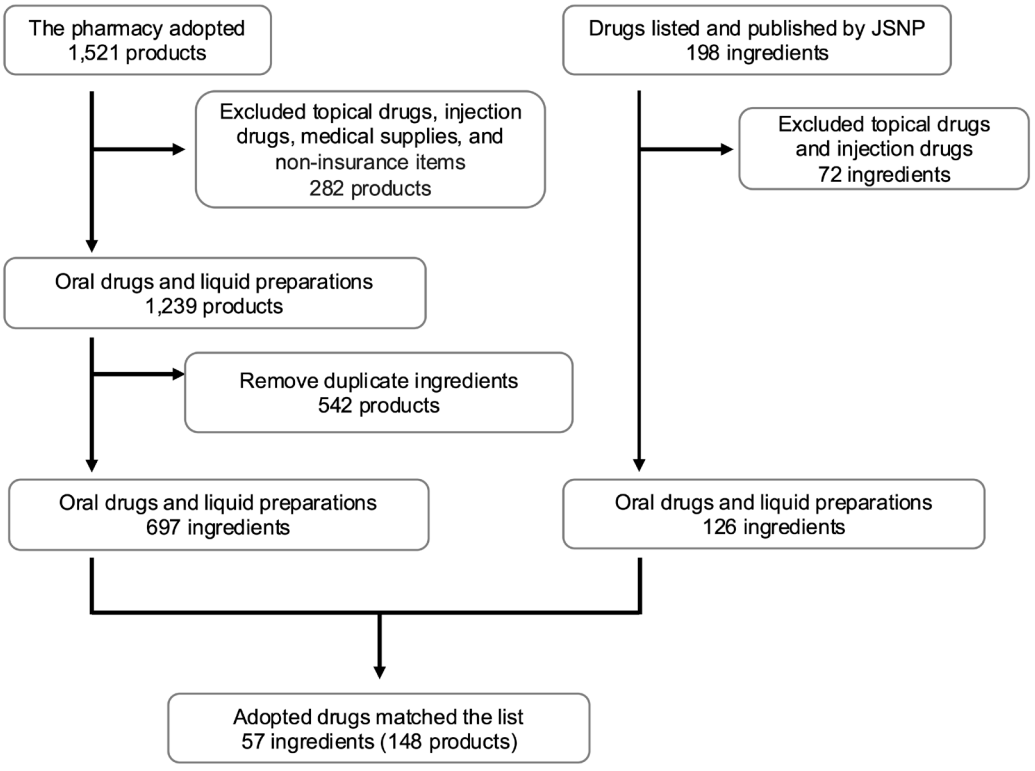


**Figure 1. Scheme of the process of comparing pharmacy-adopted drugs with the renal function adjustment drug list.** A scheme depicting the process of comparing drugs adopted by the pharmacy with the renal function adjustment drug list published by the JSNP. The number of products, including dosage variation, is noted for each ingredient and product name.

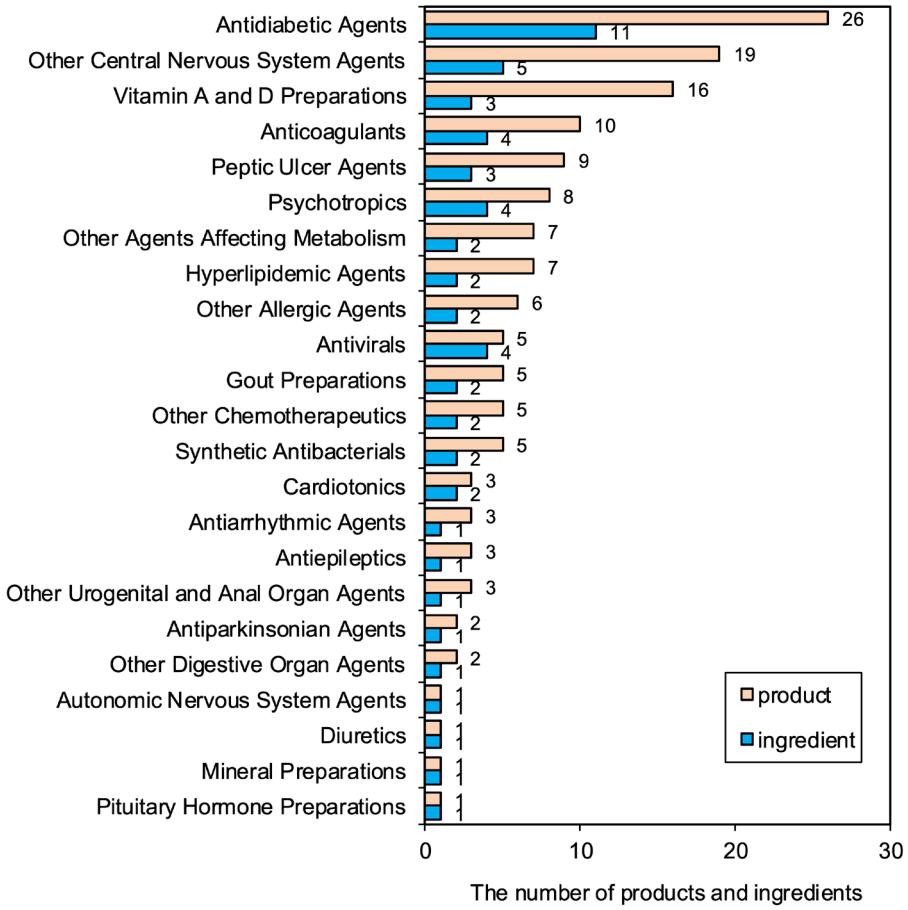


**Figure 2. The number of products and ingredients requiring dose adjustment is categorized by the therapeutic classification.** The drug classification information system published by the MHLW was used to categorize the data. A bar graph summarizes the product names and the number of ingredients in descending order for each therapeutic category.

Pharmacy after April 1, 2024, were selected in order of their visits. Medication records were collected for a total of 403 patients who met the study population criteria. Fourteen patients under 18 were excluded, as the eGFR calculation differs between adults and minors. Consequently, 389 patients were included in this study. Table 1 shows the characteristics of these 389 patients. Among them, 193 (49.6%) were male, and 196 (50.4%) were female. When categorized by age group, 221 (56.8%) were non-older adults and 168 (43.2%) were older adults. Among older adults, 89 (22.9%) were early-stage, and 79 (20.3%) were late-stage. The average number of drugs per patient was 4.4 ± 2.8, and 114 patients (29.3%) met the criteria for polypharmacy, defined as taking six or more medications. Additionally, 128 patients (32.9%) were taking at least one of the listed drugs. Among these, 8 patients (2.1%) took the highest number of listed drugs, with three drugs each.

**Table 1. Characteristics of patients included in this study (*n* = 389)**

| | *n* (%) |
|---|---|
| Gender | |
| Male | 193 (49.6) |
| Female | 196 (50.4) |
| Age | |
| 18-64 | 221 (56.8) |
| ≥ 65 | 168 (43.2) |
| Number of drugs | |
| 1-5 | 275 (70.7) |
| 6-9 | 92 (23.7) |
| ≥ 10 | 22 (5.7) |
| Number of prescriptions for listed drugs | |
| 0 | 261 (67.1) |
| 1 | 98 (25.2) |
| 2 | 22 (5.7) |
| 3 | 8 (2.1) |

### 3.3. The availability of renal function laboratory values and their sources

Table 2 presents the availability and sources of renal function laboratory values, including eGFR, BUN, and Cr. A total of 40 patients (10.3%) had at least one available laboratory value. Among these, 34 patients (8.7%) had eGFR values, which are essential for dose adjustment in pharmacies. Laboratory values came from three sources: blood test results sheets brought by patients, electronic health records accessed *via* the

**Table 3. Total number of prescriptions for listed drugs**

| Pharmacological Classification | Ingredient |
|---|---|
| Vitamin A and D Preparations (32) | Eldecalcitol (24), Alfacalcidol (5), Vitamin A (3) |
| Antidiabetic Agents (31) | Metformin (10), Glimepiride (8), Sitagliptin (6), Anagliptin/Metformin (2), Vildagliptin/Metformin (2), Alogliptin (1), Imeglimin (1), Nateglinide (1), Alogliptin/Pioglitazone (0), Pioglitazone/Metformin (0), Trelagliptin (0) |
| Peptic Ulcer Agents (19) | Famotidine (12), Cimetidine (4), Sulpiride (3) |
| Gout Preparations (13) | Allopurinol (11), Colchicine (2) |
| Other Central Nervous System Agents (11) | Pregabalin (5), Mirogabalin (4), Acamprosate (0), Memantine (1), Tiapride (1) |
| Psychotropics (11) | Duloxetine (5), Mirtazapine (3), Venlafaxine (2), Risperidone (1) |
| Other Allergic Agents (10) | Levocetirizine (9), Fexofenadine/Pseudoephedrine (1) |
| Hyperlipidemia Agents (9) | Fenofibrate (7), Bezafibrate (2) |
| Anticoagulants (8) | Edoxaban (5), Apixaban (1), Dabigatran (1), Rivaroxaban (1) |
| Synthetic Antibacterials (7) | Sitafloxacin (4), Levofloxacin (3) |
| Other Agents Affecting Metabolism (4) | Methotrexate (2), Risedronic acid (2) |
| Other Urogenital and Anal Organ Agents (4) | Tadalafil (4) |
| Antivirals (3) | Valacyclovir (3), Entecavir (0), Famciclovir (0), Oseltamivir (0) |
| Antiarrhythmic Agents (2) | Atenolol (2) |
| Antiepileptics (1) | Levetiracetam (1) |
| Pituitary Hormone Preparations (1) | Desmopressin (1) |
| Antiparkinsonian Agents (0) | Amantadine (0) |
| Autonomic Nervous System Agents (0) | Distigmine (0) |
| Cardiotonics (0) | Digoxin (0), Metildigoxin (0) |
| Diuretics (0) | Acetazolamide (0) |
| Mineral Preparations (0) | Potassium Chloride (0) |
| Other Chemotherapeutics (0) | Fluconazole (0), Trimethoprim/Sulfamethoxazole (0) |
| Other Digestive Organ Agents (0) | Metoclopramide (0) |

**Table 2. Availability of renal function laboratory values and their sources**

| | *n* (%) |
|---|---|
| Not available | 349 (89.7) |
| Available | 40 (10.3) |
| Laboratory Report | 30 (75.0) |
| Specific Health Checkup information | 7 (17.5) |
| Prescription | 3 (7.5) |

My Number System, and prescriptions containing laboratory values. Community pharmacists scanned these documents and attached them to medication records. The most common source was blood test result sheets. Thirty patients (75.0%) provided laboratory values to the pharmacy through this method.

3.4. Prescription of listed drugs

Table 3 provides a summary of drugs requiring dosage adjustment according to renal function, categorized by therapeutic class and specific ingredients. The listed drugs were prescribed a total of 166 times. Drugs from 16 therapeutic categories were prescribed. Vitamins A and D were the most frequently prescribed category, administered to 32 patients. The most frequently prescribed drug was Eldecalcitol (24 times), followed by Famotidine (12 times) and Allopurinol (11 times). Among the listed drugs, antidiabetic agents represented the category with the highest number of entries (Figure 2). Antidiabetic agents were prescribed to 31 patients, making them the second most frequently prescribed therapeutic category.

3.5. Characteristics of patients who were prescribed the listed drugs

A total of 389 patients were divided into two groups: those prescribed listed drugs (prescribed group, 128 patients) and those not prescribed these drugs (non-prescribed group, 261 patients) (Table 1). Renal function laboratory values were available for 15.6% of patients in the prescribed group (20/128) and 7.7% in the non-prescribed group (20/261), with a significantly higher proportion in the prescribed group ($\chi^2$ test, $p = 0.015$) (Figure 3A). However, despite the high risk of overdose in the prescribed group due to renal impairment, approximately 85% of these patients lacked renal function laboratory data. Additionally, the relationship between listed drug prescriptions and age is shown in Figure 3B. Among the 128 patients in the prescribed group, 54.7% (70 patients) were older adults, compared to 37.5% (98/261) in the non-prescribed group. The proportion of older adult patients was significantly higher in the prescribed group ($\chi^2$ test, $p = 0.001$). Additionally, the medical institutions that issued laboratory values were categorized. Among the 33 patients, clinics were the most common source (18 patients). A similar pattern was observed for test sheets. Conversely, all prescriptions accompanied by laboratory values were issued by university hospitals (Figure 3C). Access to electronic health check-up records (7 patients) was excluded, as it was unrelated to the prescribing institution.

### 3.6. Association between eGFR and listed drug prescription in older adults

Among the various indicators of renal function, eGFR is the most important and can be directly used for dose adjustment in cases of renal impairment. In the prescribed group, 19 of 128 patients (14.8%) provided their eGFR to the pharmacy, compared to 15 of 261 patients (5.7%) in the non-prescribed group, showing a significant difference ($\chi^2$ test, $p = 0.003$) (Figure 4A). The relationship between the availability of eGFR and age is shown in Figure 4B. Among the 34 patients who provided their eGFR, 21 (61.8%) were older adults. In contrast, among the 355 patients without their eGFR data, 147 (41.4%) were older adults, representing a significantly lower proportion than those who provided their eGFR ($\chi^2$ test, $p = 0.022$). Figure 4C shows the actual eGFR values for 34 patients, divided into non-older adults (*13*) and older adults (*21*). The mean eGFR in non-older adults was 76.0 ± 16.0 mL/min/1.73m², compared to 64.1 ± 17.5 mL/min/1.73m² in older adults, but the difference was not statistically significant (two-sided *t*-test, $p = 0.063$). Prior to this analysis, it was confirmed that there was no significant deviation from normality or inequality of variances, ensuring the appropriateness of the parametric test. These findings indicate that older adults tend to have lower renal function than non-older adults and that renal function declines with age, necessitating caution in drug dosage. Furthermore, among the 34 patients with available eGFR, 9 (2 non-older adults and 7 older adults) had eGFR values below 60 mL/min/1.73m², meeting the diagnostic criteria for CKD. These nine patients represent a subgroup requiring particular caution in drug dosing (below the dotted lines).

### 3.7. Evaluation of acute kidney injury due to Single, Double, Triple Whammy

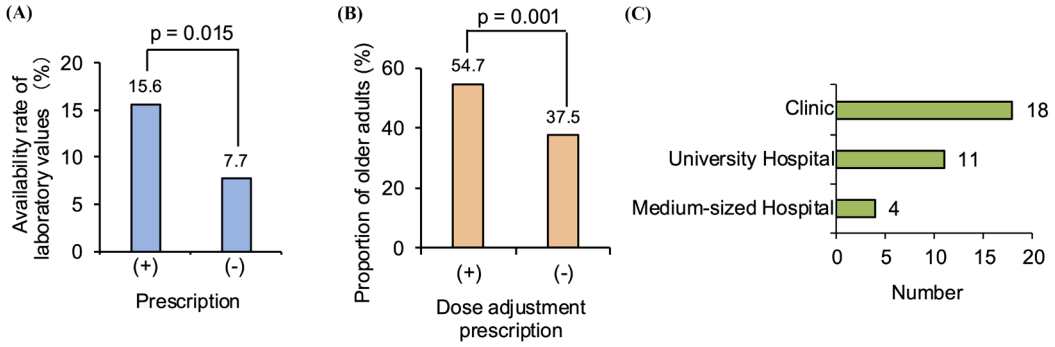


**Figure 3. The relationship between the availability of renal function laboratory values, medications requiring dose adjustments, and age. (A)** Comparison of the proportion of patients with available laboratory values between those prescribed at least one drug requiring dosage adjustment and those not prescribed such drugs. The p-values calculated using Pearson's chi-squared test are shown in the figure. **(B)** Comparison of the proportion of older adults between those prescribed at least one drug requiring dosage adjustment and those not prescribed such drugs. **(C)** The healthcare institutions that issued prescriptions with laboratory values and those that provided clinical laboratory values are categorized by their size and depicted in a bar graph. Seven individuals are not included in the figure due to their specific health check-up information being obtained through the company listed on their "My Number Card."

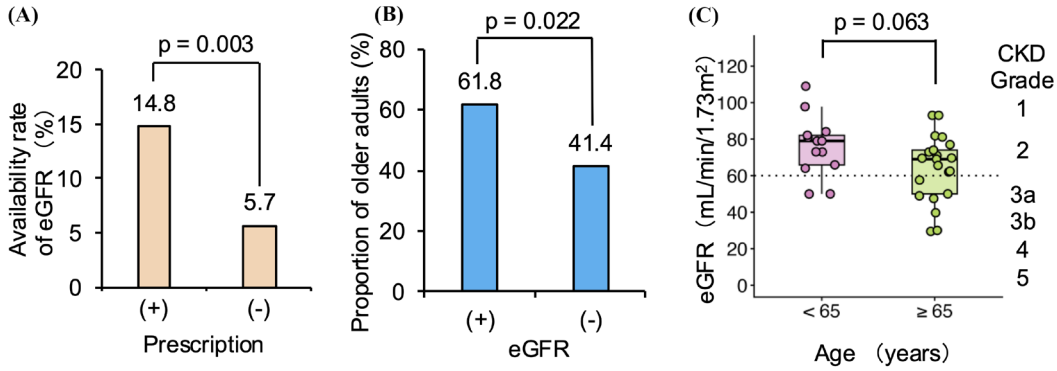


**Figure 4. The relationship between the availability of eGFR, medications requiring dose adjustments, and age. (A)** Comparison of the proportion of patients with eGFR values between those prescribed at least one drug requiring dosage adjustment and those not prescribed such drugs. The p-values calculated using Pearson's chi-squared test are shown in the figure. **(B)** The proportion of older adults in groups with and without available eGFR values is depicted in the bar graph. **(C)** The eGFR values between older adults and non-older adults were compared using a box plot. The dotted line indicates the threshold requiring dosage adjustment for most listed drugs (eGFR < 60 mL/min/1.73 m²). The CKD risk classification based on eGFR values ranged from Grade 1 to 5. The p-values calculated using the *t*-test are shown in the figure.

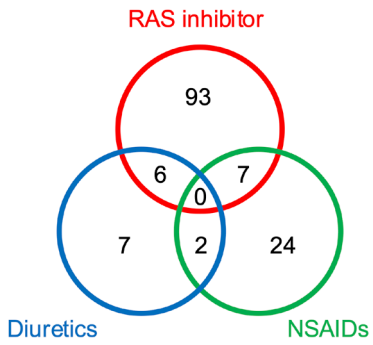


**Figure 5. Number of patients with Single, Double, Triple Whammy Risk.** The number of patients prescribed RAS inhibitors, diuretics, and NSAIDs is counted and presented using a Venn diagram. The numbers are based on the prescription of 389 patients, including those who were prescribed multiple drugs. A total of 250 patients who were not prescribed any of these drugs are excluded from the diagram.

Use of one, two, or all drug classes—RAS inhibitors, diuretics, and NSAIDs—was defined as Single, Double, and Triple Whammy, respectively. Notably, except for diuretics acetazolamide, RAS inhibitors, and NSAIDs were not included among the listed drugs. The number of patients classified as Single, Double, and Triple Whammy was 124 (31.9%), 15 (3.9%), and 0 (0%), respectively (Figure 5). The largest group consisted of 250 patients (64.3%) who did not fall into any of these categories. Among patients classified as Single or Double Whammy, ARBs were commonly used as RAS inhibitors—for example, azilsartan (28 patients) and telmisartan (27 patients). Among diuretics, furosemide—a loop diuretic—was the most frequently used and prescribed to seven patients. Loxoprofen sodium hydrate, the most commonly used NSAID in Japan, was prescribed to 18 patients. Among patients classified as Double Whammy, the most common drug combination was RAS inhibitors and NSAIDs (seven patients), followed by RAS inhibitors and diuretics (six patients), diuretics and NSAIDs (two patients). No patients were prescribed RAS inhibitors, diuretics, and NSAIDs concurrently by different medical institutions.

## 4. Discussion

Renal function laboratory values are unfamiliar and not widely recognized among the public. However, these values are critical for the safe use of medications, as their absence can lead to life-threatening consequences. In August 2011, Boehringer Ingelheim Japan issued a safety alert, commonly known in Japan as a 'Blue Letter' due to its distinctive blue paper, for the anticoagulant Pradaxa Capsule. The alert reported 139 cases of severe bleeding potentially related to the drug, including 15 fatalities. Among these, 49 cases involved renal impairment, and 22 patients had contraindicated clearance levels (eCCr < 30 mL/min). Access to Cr values in pharmacies might have enabled pharmacists to assess the drug's appropriateness. Previous studies have reported that 88.4% of community pharmacists find it difficult to obtain patients' laboratory values (*9*). Therefore, this study aimed to assess the availability and consideration of renal function laboratory values in Japanese community pharmacies. A list of drugs requiring dosage adjustment is publicly available from the JSNP and is widely used in both hospitals and community pharmacies in Japan. In this study, we examined the extent to which listed drugs are included in Keio University Community Pharmacy and identified the most common therapeutic categories. Excluding topical medications, 57 ingredients accounting for 148 of 1,239 adopted products (12%), were included in the list of drugs requiring renal function laboratory values for prescription auditing (Figure 1). These drugs fell into 16 categories, classified by therapeutic type (Figure 2). The community pharmacy in this study receives prescriptions from over 100 medical institutions monthly, covering a wide geographical area and diverse medical specialties. Therefore, the data in this study are considered generalizable. Among the listed drugs, antidiabetic agents were most prevalent, comprising 11 ingredients (26 products) (Figure 2). Additionally, antidiabetic agents such as metformin, glimepiride, and sitagliptin were prescribed 31 times among the 389 patients, accounting for approximately 8.0% (Table 3). This number of prescriptions ranked them the second most frequently prescribed category after vitamin A and D agents. A study using Japan's medical insurance data reported that approximately 20% of patients with diabetes experienced rapid renal function decline, 1.2 times higher than patients without diabetes (*13*). Furthermore, since diabetic nephropathy is the most common underlying condition in patients on dialysis, monitoring renal function in individuals with diabetes is essential (*14*). Based on the clinical prioritization perspective, antidiabetic agents should be prioritized for information sharing, as they are frequently prescribed and require careful management of renal function. In this study, eldecalcitol was the most frequently prescribed drug (Table 3). Furthermore, in the Japanese Adverse Drug Event Report (JADER) database, eldecalcitol was the second most frequently suspected drug for acute kidney disease, following valacyclovir hydrochloride (*15*). Therefore, it is crucial to monitor not only calcium levels but also renal function. Additionally, osteoporosis affects 15.9 million people in Japan, and its prevalence increases with age (*16*). In Japan's aging society, where community pharmacies play an essential role, this drug should continue to be carefully monitored.

Nearly half of pharmacists identified the lack of patients' renal function data as a barrier to dose adjustment (*6*). In this study, it was shown that the proportion of renal function laboratory values obtained from community pharmacies was 10.3% (40/389; Table 2). A nationwide survey in Japan reported a

renal function verification rate of 5.5% in community pharmacies, which was numerically close to that observed in the present study (*17*). This finding highlights the lack of renal function information, which poses a significant barrier to appropriate dose adjustment. Currently, structured systems for real-time data sharing between clinics and community pharmacies are not widely established in the Japanese healthcare system. Consequently, the most common and practical method for pharmacists to obtain laboratory data is for patients to voluntarily present paper-based reports from medical institutions. To overcome this barrier and facilitate proactive recommendations to physicians, utilizing digital infrastructure is essential. While specific health check-up data can currently be accessed *via* the national ID system (My Number Card), integrating clinical laboratory values into more accessible digital platforms, such as electronic medication notebooks or Personal Health Records, is required. This would allow pharmacists seamless access to patient data without placing an extra communication burden on physicians. Furthermore, the obtained eGFR values revealed that older patients had lower values than non-older patients (Figure 4C). It is well known that kidney function declines with age, and a similar tendency was observed in our study (*4*). These findings indicate that pharmacists should pay particular attention to reviewing laboratory values in older adults. The proportion of older adults was significantly higher in the group with available eGFR values compared to the group without (Figure 4B). This finding suggests that pharmacists may already be actively seeking laboratory values for older adults with expected renal decline. Moreover, as the number of regularly used drugs increases with age, older adult patients are more likely to have chronic diseases and undergo routine medical visits and blood tests, making it easier to obtain laboratory values (*18*). While many community pharmacists find it difficult to obtain patients' laboratory values, findings suggest that they attempt to acquire such values for patients requiring special caution (*4,6*). Currently, including laboratory values on prescriptions is not mandatory in Japan; rather, it is positioned as an advanced initiative by specific institutions, such as university hospitals, to enhance information sharing with pharmacies. Although patients often receive their test results in printed form, the majority do not fully understand the importance of having pharmacists review these values. Regarding patient characteristics, laboratory values are easier to obtain from a specific group of patients who proactively wish to review their results together with healthcare professionals. To shift from this limited access to comprehensive confirmation, the widespread adoption of electronic prescriptions and enhanced integration of laboratory values and diagnoses will be a potential solution. In the context of real-world practice, more immediate and realistic improvement strategies include hospitals and clinics attaching key laboratory values directly to prescriptions and pharmacies utilizing standardized interview templates during medication history taking to systematically screen the available test results. Strengthening local collaboration and internal workflow standardization will be vital until a fully integrated digital infrastructure is established. The concomitant use of RAS inhibitors, diuretics, and NSAIDs is referred to as the "Triple Whammy." This combination synergistically reduces renal blood flow and glomerular filtration pressure, ultimately leading to kidney injury. The National Institute of Health Sciences (NIHS) issued repeated Drug Safety Information warnings in 2003, 2006, and 2013 regarding the "Triple Whammy." They highlighted that this combination should be avoided due to its high risk of causing kidney injury, especially in older adults, individuals with existing renal impairment, and those with hydration issues. In particular, their 2013 warning spotted that the combination of three drugs poses a higher risk of causing acute kidney injury compared to the combination of two drugs. In this study, no patients met the criteria for Triple Whammy (Figure 5). Additionally, a previous study by Imai *et al*. reported that only 0.3% of patients met the criteria for Triple Whammy (*19*). However, a study using the JADER database reported that approximately half of the patients who developed AKI were taking the Triple Whammy drugs, and another report stated that the risk of AKI increases 1.3-fold due to Triple Whammy (*20,21*). Therefore, community pharmacies must remain vigilant. This study showed that all 15 patients who met the criteria for Double Whammy were prescribed two drugs by the same medical institution. For these patients, particular attention should be paid to the possibility of a third drug being prescribed by a different department. Furthermore, NSAIDs are widely used as over-the-counter (OTC) drugs in Japan. In this study, six patients were using RAS inhibitors and diuretics. Community pharmacists should consider not only prescriptions from medical institutions but also comprehensive patient information. Reports suggest that pharmacists' access to clinical laboratory data improves medication accuracy management, enabling community pharmacists to contribute to safe pharmacotherapy (*22*). A study involving 199 CKD patients reported that active vitamin D preparations were significantly more common in the adverse reaction group, leading some to suggest that this should be called the "Fourth Whammy (*23*)." In our study, eldecalcitol was the most commonly used preparation (Table 3). This finding indicates that further investigation is necessary, including monitoring the course of patients taking this drug.

**5. Limitations**

This study has several limitations. First, it is a retrospective study based on medication histories, which may omit prescribed drugs and laboratory

values. Additionally, although a drug is recorded in the medication history, it is unclear whether the patients actually took it. Second, some renal function laboratory values may not reflect the most current data. In particular, laboratory values from the Specific Health Check-up accessed *via* the My Number system require time to be updated. Therefore, the laboratory values used in this study may not accurately reflect patients' current renal function. Consequently, temporal changes in renal function must be considered when interpreting the study's results. Finally, only prescribed drugs data from medical histories were collected, so information on temporarily used OTC drugs may be missing.

## 6. Conclusion

This study suggested that community pharmacists should obtain renal function laboratory values for older adults, who are more likely to be prescribed medications requiring dose adjustment. However, only about 10% of cases had available laboratory values. Antidiabetic agents and vitamin A and D agents were commonly prescribed, underscoring the importance of verifying renal function in these cases. Although no patients met the criteria for Triple Whammy, 139 out of 389 patients (35.7%) were considered at risk. Furthermore, it is desirable that national guidelines for pharmacy-based point-of-care testing be expanded in the future to include renal function parameters. This would enable pharmacists to directly assess patient status and provide evidence-based recommendations. In the future, it will be necessary to proactively suggest the need for testing to physicians. Creating an environment that enables community pharmacists to access patients' laboratory values and proper medication management, including not only prescribed drugs but also OTC drugs, can be ensured, and community pharmacists can contribute to safe pharmacotherapy.

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## References

1. Chen TK, Knicely DH, Grams ME. Chronic kidney disease diagnosis and management: A review. JAMA. 2019; 322:1294-1304.
2. Hill NR, Fatoba ST, Oke JL, Hirst JA, O'Callaghan CA, Lasserson DS, Hobbs FD. Global prevalence of chronic kidney disease – A systematic review and meta-analysis. PLOS One. 2016; 11:e0158765.
3. Xie Y, Bowe B, Mokdad AH, Xian H, Yan Y, Li T, Maddukuri G, Tsai CY, Floyd T, Al-Aly Z. Analysis of the Global Burden of Disease study highlights the global, regional, and national trends of chronic kidney disease epidemiology from 1990 to 2016. Kidney Int. 2018; 94:567-581.
4. Imai E, Horio M, Yamagata K, Iseki K, Hara S, Ura N, Kiyohara Y, Makino H, Hishida A, Matsuo S. Slower decline of glomerular filtration rate in the Japanese general population: A longitudinal 10-year follow-up study. Hypertens Res. 2008; 31:433-441.
5. Imai E, Horio M, Iseki K, *et al.* Prevalence of chronic kidney disease (CKD) in the Japanese general population predicted by the MDRD equation modified by a Japanese coefficient. Clin Exp Nephrol. 2007; 11:156-163.
6. Zafar R, Rehman IU, Shah Y, Ali Z, Ming LC, Khan TM. Knowledge, attitude and perceptions of pharmacists regarding renal dose adjustment among chronic kidney disease patients in Pakistan. J Pharm Policy Pract. 2023; 16:102.
7. Wada R, Takeuchi J, Nakamura T, Sonoyama T, Kosaka S, Matsumoto C, Sakuma M, Ohta Y, Morimoto T. Clinical decision support system with renal dose adjustment did not improve subsequent renal and hepatic function among inpatients: The Japan adverse drug event study. Appl Clin Inform. 2020; 11:846-856.
8. Kappel J, Calissi P. Nephrology: 3. Safe drug prescribing for patients with renal insufficiency. CMAJ. 2002; 166:473-477.
9. Kondo Y, Ishitsuka Y, Shigemori E, Irikura M, Kadowaki D, Hirata S, Maemura T, Irie T. Awareness and current implementation of drug dosage adjustment by pharmacists in patients with chronic kidney disease in Japan: A web-based survey. BMC Health Serv Res. 2014; 14:615.
10. The Japanese Society of Nephrology and Pharmacotherapy. Dosage recommendations for drugs that require the most attention in renal impairment. 37th ed. 2024. *https://www.jsnp.org/files/dosage_recommendations_37.pdf* (Accessed March 31, 2026).
11. Oni L, Hawcutt DB, Turner MA, Beresford MW, McWilliam S, Barton C, Park BK, Murray P, Wilm B, Copple I, Floyd R, Peak M, Sharma A, Antoine DJ. Optimising the use of medicines to reduce acute kidney injury in children and babies. Pharmacol Ther. 2017; 174:55-62.
12. Harężlak T, Religioni U, Szymański FM, Hering D, Barańska A, Neumann-Podczaska A, Allan M, Merks P. Drug Interactions Affecting Kidney Function: Beware of Health Threats from Triple Whammy. Adv Ther. 2022; 39:140-147.
13. Fujii M, Ohno Y, Ikeda A, Godai K, Li Y, Nakamura Y, Yabe D, Tsushita K, Kashihara N, Kamide K, Kabayama M. Current status of the rapid decline in renal function due to diabetes mellitus and its associated factors: Analysis using the National Database of Health Checkups in Japan. Hypertens Res. 2023; 46:1075-1089.
14. Nitta K, Goto S, Masakane I, Hanafusa N, Taniguchi M, Hasegawa T, Nakai S, Wada A, Hamano T, Hoshino J, Joki N, Abe M, Yamamoto K, Nakamoto H. Annual dialysis data report for 2018, JSDT Renal Data Registry: survey methods, facility data, incidence, prevalence, and mortality. Ren Replace Ther. 2020; 6:41.

15. Hosohata K, Inada A, Oyama S, Furushima D, Yamada H, Iwanaga K. Surveillance of drugs that most frequently induce acute kidney injury: A pharmacovigilance approach. J Clin Pharm Ther. 2019; 44:49-53.
16. Yoshimura N, Iidaka T, Horii C, Muraki S, Oka H, Kawaguchi H, Nakamura K, Akune T, Tanaka S. Trends in osteoporosis prevalence over a 10 year period in Japan: The ROAD study 2005–2015. J Bone Miner Metab. 2022; 40:829-838.
17. Kondo Y, Shikamura Y, Suzuki M, Takahashi M, Kawakami E, Hashiba H, Miyazaki C. Renal function ascertainment rate of patients at community pharmacies in Japan: a national survey. Ren Replace Ther. 2025; 11:22.
18. Moffet HH, Parker MM, Sarkar U, Schillinger D, Fernandez A, Adler NE, Adams AS, Karter AJ. Adherence to laboratory test requests by patients with diabetes: the Diabetes Study of Northern California (DISTANCE). Am J Manag Care. 2011; 17:339-344.
19. Imai S, Momo K, Kashiwagi H, Miyai T, Sugawara M, Takekuma Y. A cross-sectional exploratory survey on occurrence of triple-whammy prescription pattern in Japan. Int J Clin Pharm. 2020; 42:1369-1373.
20. Kunitsu Y, Hira D, Morikochi A, Ueda T, Isono T, Morita S, Terada T. Time until onset of acute kidney injury by combination therapy with "Triple Whammy" drugs obtained from Japanese Adverse Drug Event Report database. PLOS One. 2022; 17:e0263682.
21. Lapi F, Azoulay L, Yin H, Nessim SJ, Suissa S. Concurrent use of diuretics, angiotensin converting enzyme inhibitors, and angiotensin receptor blockers with non-steroidal anti-inflammatory drugs and risk of acute kidney injury: nested case-control study. BMJ. 2013; 346:e8525.
22. Kestin R, Howe A, Moose J, Marciniak MW, Rhodes LA. Impact of health information exchange access on medication management recommendations in a community-based pharmacy setting. J Am Pharm Assoc (2003). 2024; 64:102104.
23. Narisue M, Sugimoto Y, Hirano F, Nakatsukasa R, Miyazaki K, Otsubo T, Nakashima M, Hirata S. Survey of prescriptions for triple whammy drug combinations with vitamin D as a possible fourth whammy. Int J Clin Pharmacol Ther. 2023; 61:8-15.

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# Metabolic and endocrinological effects of weekly growth hormone replacement therapy with somapacitan in patients with adult growth hormone deficiency after switching from daily growth hormone replacement therapy: A real-world exploratory cohort study

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**SUMMARY**: The metabolic and endocrinological effects in patients with adult growth hormone deficiency (AGHD) who switched from daily growth hormone (GH) replacement therapy to weekly GH replacement therapy with somapacitan were evaluated and observed over a long follow-up period. Patients were included only if their medical treatments, aside from GH replacement therapy, remained unchanged. Metabolic and endocrinological parameters were assessed at the time of switching, and at 1- and 2-year follow-up after switching from daily GH replacement therapy to weekly GH replacement therapy with somapacitan. The results showed that the body mass index (BMI), fasting plasma glucose (FPG), aspartate transaminase (AST), and triglyceride (TG) levels at 1 year after switching significantly improved compared with those at the time of switching (each $P < 0.025$). At 2 years, the homeostasis model assessment of insulin resistance (HOMA-IR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and alanine aminotransferase (ALT), as well as BMI, FPG, TG, and AST significantly improved compared with those observed at the time of switching (each $P < 0.025$). In addition, improvement in HOMA-IR at 2 years after switching was significantly associated with improvement in AST. Switching to GH replacement therapy did not affect endocrinological parameters. These findings, which were revealed by the present real-world exploratory cohort study, indicate that weekly GH replacement therapy with somapacitan may confer more beneficial effects than daily GH replacement therapy.

***Keywords***: Growth hormone, adult growth hormone deficiency, somapacitan

## 1. Introduction

Adult growth hormone deficiency (AGHD) is an anterior pituitary hormonal deficiency (*1,2*). AGHD causes fatty liver, metabolic dysfunction-associated steatohepatitis (MASH), metabolic dysfunction-associated steatotic liver disease (MASLD), increased visceral adiposity, osteoporosis, impaired quality of life (QOL), poor concentration, inattention, coronary artery disease, and heart failure (*3-10*). Accordingly, AGHD may be associated with increased mortality. Growth hormone (GH) replacement therapy has been reported to reduce mortality in patients with AGHD (*10*). Therefore, GH replacement therapy is essential for patients with AGHD. Globally, daily GH replacement therapy had long been the only available treatment for patients with AGHD. It is well known that GH concentrations in the blood are normally high at midnight and are low during the day, whereas GH concentrations in patients with AGHD are extremely low throughout the day. Thus, patients with AGHD commonly self-inject GH formulations nightly (between 7:00 pm and 8.00 pm). However, blood GH concentrations of patients with AGHD during the daytime remain severely low compared with those in normal subjects, as the duration of daily GH formulations is less than 12 h (*11,12*). Recently, patients with AGHD

have been able to use somapacitan, the only available weekly GH formulation. Nevertheless, the prolonged duration of somapacitan exceeds 1 week, and the effects of this formulation are maintained throughout the day. Considering the differences in duration, metabolic and endocrinological parameters may differ between daily GH replacement therapy and weekly GH replacement therapy with somapacitan. Previously, we reported a real-world pilot study that revealed that the body mass index (BMI), homeostasis model assessment of insulin resistance (HOMA-IR), fasting plasma glucose (FPG), and liver function parameters were significantly improved 6 months after switching from daily GH replacement therapy to weekly GH replacement therapy with somapacitan compared with those observed at the time of switching (*13*). In the present study, we measured and compared the metabolic and endocrinological parameters at the time of switching from daily GH replacement to weekly GH replacement therapy with somapacitan, and at 1- and 2-year follow-up after switching in patients with AGHD.

## 2. Patients and Methods

### 2.1. Ethical approval of the study protocol

This real-world exploratory cohort study protocol was approved by the ethics review committees of Fukuoka University (Fukuoka, Japan. Approval number: C25-09-006). Written informed consent was obtained from all participants in the study. This study was conducted in accordance with the principles of the Declaration of Helsinki.

### 2.2. Study participants

We investigated 12 individuals with AGHD who had been diagnosed on the basis of no or inadequate GH responses to a GH-releasing peptide-2 test, insulin tolerance test, or arginine test at Fukuoka University Chikushi Hospital and Nagasaki Prefecture Iki Hospital (*13-15*). All patients had received daily GH replacement therapy for over 2 years and switched to weekly GH replacement therapy with somapacitan, which was continued for 2 years. No medical treatments other than GH replacement therapy were changed during the evaluation period.

### 2.3. Methods and disease definitions

We administered somapacitan for 2 years in patients with AGHD who had previously received daily GH replacement therapy. The starting dose at the time of switching were 1.5 mg/week for adults aged 18-60 years and 1.0 mg/week for patients aged > 60 years, in accordance with recommendation from a phase 3 trial in Japan (REAL Japan) (*16*). Dose titration was performed according to the insulin-like growth factor 1 (IGF1) levels, as described in our previous study (*13*). The following variables were examined at the time of switching, and at 1- and 2-year follow-up after switching: parameters of glucose control (glycated hemoglobin (HbA1c), fasting plasma glucose (FPG), fasting insulin, homeostasis model assessment of insulin resistance (HOMA-IR), and homeostasis model assessment of β-cell function (HOMA-β), markers of lipid metabolism (low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG), liver function parameters (aspartate transaminase (AST), alanine transaminase (ALT), and gamma-glutamyl transferase (γ-GTP)), estimated glomerular filtration rate (eGFR), systolic blood pressure (SBP), diastolic blood pressure (DBP), and BMI. Blood samples were obtained after overnight fasting.

Regarding assessment of insulin secretion, HOMA-β was calculated using the following formula:

$$\text{HOMA-}\beta = 360 \times \text{fasting insulin } (\mu\text{U/mL})/(\text{FPG (mg/dL)} - 63)$$

Regarding assessment of insulin resistance, HOMA-IR was calculated using the following formula:

$$\text{HOMA-IR} = \text{FPG (mg/dL)} \times \text{fasting insulin } (\mu\text{U/mL})/405$$

Endocrinologically, anterior pituitary hormones and related hormones (adrenocorticotropic hormone (ACTH), cortisol, thyroid-stimulating hormone (TSH), free thyroxine 4 (T4), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone [male]/estradiol [female]) were measured at the time of switching, and at 1 and 2 years after switching. ACTH deficiency was diagnosed by a combination of reduced ACTH and cortisol levels in the morning and no or inadequate changes in ACTH or cortisol levels after a corticotropin-releasing hormone test. TSH deficiency was diagnosed on the basis of a combination of reduced TSH levels, no or inadequate changes in TSH levels after a thyrotropin-releasing hormone test, and existing secondary hypothyroidism. LH or FSH deficiency was diagnosed on the basis of a combination of reduced LH or FSH levels, no or inadequate changes in LH or FSH levels after an LH-releasing hormone test, and existing secondary hypogonadism. Central diabetes insipidus was diagnosed by a combination of increased urinary volume, low urinary osmolarity, low antidiuretic hormone (ADH) levels compared with serum osmolarity, no or inadequate changes in ADH levels after a water restriction test or 5% NaCl loading test, and increased ADH levels with decreased urinary volume after 1-desamino-8-D-arginine vasopressin administration (*13-15*).

No medical treatments other than GH replacement therapy were changed during the study period.

2.4. Statistical analyses

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using Stata SE v.16 (StataCorp 2019, Stata statistical software Release 16. [College Station, TX: Stata Corp LLC]). A paired *t*-test with Dunnett's correction was used to compare the means of laboratory measurements between the time of switching and 1 year after switching, as well as between the time of switching and 2 years after switching. *P*-values < 0.025 were considered significant. Associations between improvements in HOMA-IR and other parameters were examined using univariate regression analysis, and *P*-values < 0.05 were considered statistically significant.

## 3. Results

Table 1 presents the baseline characteristics of the patients enrolled in the present study. All patients had AGHD and were receiving daily GH replacement therapy. The mean age was 60.3 ± 18.9 years, and nine patients were female. The BMI was 26.4 ± 5.1. Endocrinologically, 75.0%, 58.3%, 50.0%, and 50.0% of the patients exhibited ACTH, TSH, LH, and FSH deficiencies, respectively. A total of 8.3% of patients had central diabetes insipidus. In addition, hydrocortisone, levothyroxine, human chorionic gonadotrophin/human menopausal gonadotropin (HCG/HMG), and testosterone/estrogen, and desmopressin replacement therapy were administered in 75.0, 66.7, 8.3, 0.0, and 8.3% of patients, respectively (one patient had primary hypothyroidism rather than TSH deficiency and was administered levothyroxine). As a result of titration, IGF1 values at 1 and 2 years after switching to somapacitan were comparable to those at the time of switching (103.5 ± 43.8 at switching *vs.* 95.2 ± 43.2 ng/mL at 1 year, *P* = 0.121; and 101.2 ± 35.2 ng/mL at 2 years, *P* = 0.352). Moreover, the IGF1 values of all patients at the time of switching to somapacitan and at 1 and 2 years after switching were between -1 SDS and +1 SDS. The mean dose of daily GH replacement at the time of switching was 0.20 ± 0.07 mg/day, and the dose of somapacitan at 1 and 2 years after switching was 1.60 ± 0.49/1.88 ± 0.61 mg/week, respectively (Table 2).

Table 3 shows the changes achieved in clinical, metabolic, and endocrinological parameters. In terms of glucose tolerance, FPG was significantly improved at 1 year after switching compared with values at the time of switching (102.8 ± 19.5 *vs.* 97.3 ± 20.3 mg/dL, *P* = 0.024). HbA1c, fasting insulin, HOMA-IR, and HOMA-β did not improve from the time of switching to 1 year after switching (HbA1c: 6.2 ± 0.5 *vs.* 6.2 ± 0.6 %, *P* = 0.403, fasting insulin: 11.6 ± 7.5 *vs.* 8.3 ± 7.2 μU/mL, *P* = 0.028, HOMA-IR: 2.8 ± 1.6 *vs.* 2.0 ± 1.7 , *P* = 0.029, HOMA-β: 137.8 ± 132.3 *vs.* 105.4 ± 92.3, *P* = 0.080). Meanwhile, HOMA-IR and FPG significantly improved from the time of switching to 2 years after switching (HOMA-IR: 2.8 ± 1.6 *vs.* 2.1 ± 1.1, *P* = 0.024, FPG: 102.8 ± 19.5 *vs.* 97.5 ± 18.9 mg/dL, *P* = 0.010). HbA1c, fasting insulin, and HOMA-β did not improve from the time of switching to 2 years after switching (HbA1c: 6.2 ± 0.5 *vs.* 6.2 ± 0.6 %, *P* = 0.330, fasting

**Table 1. Summary of patient characteristics**

| | Number of patients = 12 |
|---|---|
| Age (years, ± SD) | 60.3 ± 18.9 |
| Sex (Female/Male) | 9/3 |
| BMI (kg/m$^2$, ± SD) | 26.4 ± 5.1 |
| Hormonal deficiencies | |
| ACTH (%) | 75.0 |
| TSH (%) | 58.3 |
| LH (%) | 50.0 |
| FSH (%) | 50.0 |
| Central diabetes insipidus (%) | 8.3 |
| GH (%) | 100 |
| Replacement therapies | |
| Hydrocortisone (%) | 75.0 |
| Levothyroxine (%) | 66.7[#] |
| HCG/HMG (%) | 8.3 |
| Testosterone/Estrogen(%) | 0 |
| Desmopressin (%) | 8.3 |
| GH (%) | 100 |
| Periods of GH replacement therapy (years, ± SD) | 7.2 ± 2.9 |

[#]One patient received levothyroxine for primary hypothyroidism. ACTH, adrenocorticotropic hormone; FSH, follicle-stimulating hormone; GH, growth hormone; TSH, thyroid-stimulating hormone; HCG, human chorionic gonadotropin; HMG, human menopausal gonadotropin; LH, luteinizing hormone; SD, standard deviation.

**Table 2. Details of GH replacement therapy in our study**

| | At switching (0) | 1 year after switching (1) | *P*-value (0 *vs.* 1) | 2 years after switching (2) | *P*-value (0 *vs.* 2) |
|---|---|---|---|---|---|
| IGF-1 (ng/mL, ± SD) | 103.5 ± 43.8 | 95.2 ± 43.2 | 0.121 | 101.2 ± 35.2 | 0.352 |
| Daily dose of GH before switching (mg/day, ± SD) | 0.20 ± 0.07 | - | - | - | - |
| Weekly dose of GH 1 year after switching (mg/week, ± SD) | - | 1.60 ± 0.49 | - | - | - |
| Weekly dose of GH 2 years after switching (mg/week, ± SD) | - | - | - | 1.88 ± 0.61 | - |

Significant differences between mean values were estimated using a paired *t*-test with Dunnett's correction. *P* < 0.025 was considered significant. GH, growth hormone; IGF1, insulin-like growth factor-1; SD, standard deviation.

insulin: 11.6 ± 7.5 *vs.* 8.6 ± 4.7 μU/mL, $P = 0.045$, HOMA-β: 137.8 ± 132.3 *vs.* 108.8 ± 74.3, $P = 0.112$).

TG, a marker of lipid metabolism, significantly improved from the time of switching compared with 1 and 2 years after switching (136.5 ± 39.2 at switching *vs.* 101.3 ± 74.3 at 1 year, $P = 0.006$; and 95.6 ± 29.3 at 2 years, $P < 0.001$).

Regarding liver function, AST levels significantly improved from the time of switching to 1 and 2 years after switching (23.1 ± 3.4 at switching *vs.* 21.3 ± 4.0 at 1 year, $P = 0.019$; and 20.3 ± 3.1 at 2 years, $P = 0.006$). Meanwhile, ALT improved from the time of switching to 1 year after switching but with no significant difference (19.3 ± 5.5 *vs.* 16.7 ± 5.1, $P = 0.044$), although it improved significantly from the time of switching to 2 years after switching (19.3 ± 5.5 *vs.* 15.7 ± 6.1, $P = 0.024$).

In addition, with regard to clinical parameters, BMI significantly improved from the time of switching to 1 and 2 years after switching (26.4 ± 5.1 at switching *vs.* 25.8 ± 5.3 kg/m$^2$ at 1 year, $P = 0.015$; and 25.7 ± 5.3 kg/m$^2$ at 2 years, $P = 0.009$). In addition, SBP and DBP improved from the time of switching to 1 and 2 years after switching. Furthermore, significant changes were observed from the time of switching to 2 years after switching (SBP: 139.4 ± 17.2 *vs.* 130.2 ± 16.4 mmHg, $P = 0.023$; DBP: 80.5 ± 11.1 *vs.* 73.6 ± 9.6 mmHg, $P = 0.022$).

Regarding endocrinological parameters, no differences were observed between values at the time of switching and those at 1 and 2 years after switching for all anterior pituitary hormones and related hormones.

Besides, regression analysis showed that improvement in AST levels was significantly associated with improvement in HOMA-IR ($P = 0.026$), whereas improvement in BMI, SBP, DBP, ALT, γ-GTP, and the period of daily GH replacement therapy were not significantly associated with improvement in HOMA-IR (Table 4).

## 4. Discussion

AGHD is a pituitary hormonal deficiency, including anterior pituitary hormonal deficiency and diabetes insipidus. Patients with severe AGHD exhibit different types of metabolic disorders (*3-10*). AGHD is associated

**Table 3. Comparison of changes in metabolic and endocrinological parameters at switching from daily to weekly GH replacement therapy with somapacitan and at 1 and 2 years after switching**

| | at switching (0) | 1 year after switching (1) | *P* (0 *vs* 1) | 2 years after switching (2) | *P* (0 *vs* 2) |
|---|---|---|---|---|---|
| BMI (kg/m$^2$, ± SD) | 26.4 ± 5.1 | 25.8 ± 5.3 | 0.015* | 25.7 ± 5.3 | 0.009* |
| SBP (mmHg, ± SD) | 139.4 ± 17.2 | 132.7 ± 15.9 | 0.036 | 130.2 ± 16.4 | 0.023* |
| DBP (mmHg, ± SD) | 80.5 ± 11.1 | 75.9 ± 9.9 | 0.053 | 73.6 ± 9.6 | 0.022* |
| FPG (mg/dL, ± SD) | 102.8 ± 19.5 | 97.3 ± 20.3 | 0.024* | 97.5 ± 18.9 | 0.010* |
| HbA1c (%, ± SD) | 6.2 ± 0.5 | 6.2 ± 0.6 | 0.403 | 6.2 ± 0.6 | 0.330 |
| Fasting insulin (μIU/mL, ± SD) | 11.6 ± 7.5 | 8.3 ± 7.2 | 0.028 | 8.6 ± 4.7 | 0.045 |
| HOMA-IR (± SD) | 2.8 ± 1.6 | 2.0 ± 1.7 | 0.029 | 2.1 ± 1.1 | 0.024* |
| HOMA-β (± SD) | 137.8 ± 132.3 | 105.4 ± 92.3 | 0.080 | 108.8 ± 74.3 | 0.112 |
| AST (U/L, ± SD) | 23.1 ± 3.4 | 21.3 ± 4.0 | 0.019* | 20.3 ± 3.1 | 0.006* |
| ALT (U/L, ± SD) | 19.3 ± 5.5 | 16.7 ± 5.1 | 0.044 | 15.7 ± 6.1 | 0.024* |
| γ-GTP (U/L, ± SD) | 24.0 ± 16.1 | 21.5 ± 14.0 | 0.033 | 21.7 ± 11.7 | 0.143 |
| LDL-C (mg/dL, ± SD) | 111.2 ± 23.5 | 104.8 ± 21.6 | 0.167 | 104.3 ± 22.2 | 0.142 |
| HDL-C (mg/dL, ± SD) | 59.8 ± 8.5 | 55.8 ± 8.7 | 0.042 | 60.3 ± 10.6 | 0.427 |
| TG (mg/dL, ± SD) | 136.5 ± 39.2 | 101.3 ± 24.7 | 0.006* | 95.6 ± 29.3 | < 0.001* |
| eGFR (mL/min/1.73m$^2$) | 66.8 ± 23.9 | 66.2 ± 22.7 | 0.355 | 66.6 ± 23.1 | 0.452 |
| Na (mmol/L, ± SD) | 141.5 ± 2.7 | 141.9 ± 2.0 | 0.313 | 141.8 ± 3.1 | 0.376 |
| K (mmol/L, ± SD) | 3.9 ± 0.3 | 4.0 ± 0.7 | 0.255 | 3.8 ± 0.4 | 0.276 |
| Cl (mmol/L, ± SD) | 105.0 ± 2.1 | 104.8 ± 2.1 | 0.219 | 105.3 ± 1.9 | 0.369 |
| ACTH (pg/mL, ± SD) | 18.0 ± 21.6 | 20.1 ± 24.5 | 0.155 | 19.0 ± 23.8 | 0.215 |
| Cortisol (μg/dL, ± SD) | 5.3 ± 4.0 | 5.9 ± 4.4 | 0.229 | 5.3 ± 4.5 | 0.481 |
| TSH (μIU/mL, ± SD) | 1.9 ± 1.4 | 1.9 ± 1.4 | 0.416 | 1.8 ± 1.1 | 0.370 |
| Free T4 (ng/dL, ± SD) | 0.9 ± 0.1 | 1.0 ± 0.2 | 0.064 | 1.0 ± 0.2 | 0.053 |
| PRL (ng/mL, ± SD) | 14.6 ± 13.3 | 12.3 ± 10.5 | 0.060 | 11.4 ± 9.5 | 0.068 |
| LH (mIU/mL, ± SD) | 2.5 ± 1.6 | 3.7 ± 2.9 | 0.058 | 3.2 ± 2.6 | 0.102 |
| FSH (mIU/mL, ± SD) | 4.8 ± 3.7 | 4.7 ± 3.6 | 0.305 | 5.2 ± 3.9 | 0.028 |
| Testosterone (ng/mL, ± SD) (Male only) | 4.7 ± 3.0 | 6.1 ± 4.8 | 0.175 | 2.1 ± 1.7 | 0.203 |
| Estradiol (pg/mL, ± SD) (Female only) | 25.5 ± 61.0 | 60.2 ± 136.0 | 0.207 | 33.6 ±77.2 | 0.092 |

Significant differences between mean values were estimated using a paired *t*-test with Dunnett's correction. *$P < 0.025$ was considered significant. ACTH, adrenocorticotropic hormone; AST, aspartate transaminase; ALT, alanine aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FSH, follicle-stimulating hormone; FPG; fasting plasma glucose; γ-GTP, γ-glutamyl transferase; HbA1c, glycated hemoglobin; HOMA-β, homeostasis model assessment of β-cell function; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; LH, luteinizing hormone; PRL, prolactin; SBP, systolic blood pressure; SD, standard deviation; T4, thyroxine 4; TG, triglycerides; TSH, thyroid-stimulating hormone.

**Table 4. Relationship between improvement in HOMA-IR and other parameters**

| Regression analysis | Improvement in HOMA-IR | | |
|---|---|---|---|
| | β (SE) | 95% CI | *P*-value |
| Improvement in BMI | 2.260 (2.560) | -3.525-8.045 | 0.405 |
| Improvement in SBP | 0.487 (0.764) | -1.215-2.189 | 0.538 |
| Improvement in DBP | 0.452 (0.629) | -0.949-1.853 | 0.489 |
| Improvement in AST | 1.195 (0.456) | 0.178-2.212 | 0.026* |
| Improvement in ALT | 0.465 (0.327) | -0.263-1.194 | 0.185 |
| Improvement in γ-GTP | 0.554 (0.432) | -0.408-1.516 | 0.228 |
| Periods of daily GH replacement therapy | -3.461 (2.755) | -9.198-2.276 | 0.209 |

Significant associations between mean values were estimated using a univariate regression analysis. *$P < 0.05$ was considered significant. AST, aspartate transaminase; ALT, alanine aminotransferase; β, regression coefficient; BMI, body mass index; CI, confidence interval; DBP, diastolic blood pressure; GH, growth hormone; HOMA-IR, homeostasis model assessment of insulin resistance; CI, confidence interval; ACTH, adrenocorticotropic hormone; TSH, thyroid-stimulating hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; SBP, systolic blood pressure; SE, standard error.

with obesity, dyslipidemia, MASH/MASLD, increased insulin resistance, and increased risk of coronary heart disease (*17-19*). Considering the improvements in BMI, weekly GH replacement therapy with somapacitan may be more beneficial than daily GH replacement therapy. Regarding liver dysfunction, AST and ALT levels were significantly improved by switching from daily GH replacement therapy to weekly GH replacement therapy with somapacitan, and levels at 2 years after switching showed more effective results than those at 1 year after switching. AGHD is well-known to cause MASH/MASLD (*3,18,19*). Similarly, TG levels significantly improved after switching from daily GH replacement therapy to weekly GH replacement therapy with somapacitan. and levels at 2 years after switching were lower than those at 1 year after switching. Besides, SBP and DBP at 2 years after switching were significantly improved compared with values at the time switching, whereas SBP and DBP at 1 year after switching were improved compared with those at the time of switching treatment, but were not significantly improved. These results are consistent with those of a previous report, which revealed that GH replacement therapy could decrease SBP and DBP in patients with AGHD (*6*). Considering that improvements in the lipid profile and blood pressure reduce the risk of coronary heart disease, weekly GH replacement therapy with somapacitan may be more beneficial than daily GH replacement therapy. Furthermore, FPG levels significantly improved after switching from daily GH replacement therapy to weekly GH replacement therapy with somapacitan, and levels at 2 years after switching showed more effective results than those at 1 year after switching. In addition, HOMA-IR values at 2 years after switching were significantly improved compared with those at the time switching. Although HOMA-IR values at 1 year after switching were improved compared with those at the time of switching treatment, but were not significantly improved.

Considering these data and the lack of change in HOMA-β levels, the improvement in FPG might be due to the improvement in insulin resistance. Previously, we reported that HOMA-IR significantly improved from the time of switching treatment to the 6-month follow-up. However, no significant differences in glucose intolerance were observed between daily GH replacement therapy and weekly GH replacement therapy with somapacitan, which contrasted with the data from a phase 3 clinical trial (*20*). The differences between the trial findings and our results could be because the medical treatment of all patients in the present study did not change during this period, aside from GH formulations, although it is quite possible that medical treatment may have changed in some patients in the phase 3 trials of somapacitan (*16,21,22*). However, a post hoc analysis of one of the phase 3 studies indicated that the group receiving somapacitan had significantly lower HOMA-IR and FPG levels than those receiving daily GH formulations 32 weeks after treatment initiation, which was similar to the results of our present and previous studies. Nevertheless, several factors regulate insulin resistance. Regression analysis revealed that an improvement in AST levels was significantly associated with an improvement in HOMA-IR ($P = 0.026$). In contrast, an improvement in BMI was not significantly associated with an improvement in HOMA-IR. In addition, a previous clinical trial showed no significant differences in the body composition of patients following daily GH formulations and somapacitan treatment (*22*). Patients with AGHD may exhibit excessive hepatic insulin resistance caused by fatty liver, MASH, or MASLD (*23*). Hence, the present study revealed that weekly GH replacement therapy with somapacitan may confer more beneficial effects on improving insulin resistance by ameliorating hepatic insulin resistance than daily GH replacement therapy. In our previous study, albeit over a short follow-up period, the results indicated that improvement in HOMA-IR was significantly associated with the duration of daily GH replacement therapy before switching to weekly GH

replacement therapy with somapacitan. These findings suggest that daily GH replacement formulations could be less efficient, at least for glucose intolerance, than weekly GH replacement therapy with somapacitan. Switching to weekly GH replacement therapy with somapacitan should be considered as soon as possible if patients with AGHD are currently being treated with daily GH replacement therapy. However, in the present study, no significant association was observed between an improvement in HOMA-IR and the period of daily GH replacement therapy before switching to weekly GH replacement therapy with somapacitan. This discrepancy indicated that a longer period of weekly GH replacement therapy with somapacitan could reduce increased insulin resistance in patients who receive prolonged daily GH replacement therapy.

In the present study, we demonstrated that switching to GH replacement therapy did not affect endocrinological parameters, similar to our previous real-world pilot study (*13*). Hence, somapacitan can be used without impairing the endocrinological condition of patients.

Meanwhile, the present study has some limitations that should be acknowledged. First, the sample size was small because AGHD is a rare and intractable condition. Furthermore, we excluded patients whose medical treatments, aside from GH replacement therapy, changed during the study period. This could be the reason that the levels of HbA1c at 1 year and 2 years after switching were not significantly improved compared to those at switching. However, considering the levels of HOMA-IR were significantly improved at 2 years after switching, the levels of HbA1c could be improved with longer examination. Therefore, future studies with a larger cohort comparing groups receiving weekly GH replacement therapy with somapacitan and those receiving daily GH replacement therapy for a longer period are required. Similarly, to confirm the observed univariate association between improvements in AST and HOMA-IR, future studies with large cohort are also required. Second, we used HOMA-IR and HOMA-β as surrogate markers of insulin resistance, and insulin secretion as a substitute for an oral glucose tolerance test or hyperglycemic/hyperinsulinemic–euglycemic clamps.

Therefore, future studies with a larger cohort comparing groups receiving weekly GH replacement therapy with somapacitan and those receiving daily GH replacement therapy are required to confirm the results of the present study.

In conclusion, this study revealed that weekly GH replacement therapy with somapacitan may achieve more beneficial effects on metabolic parameters than daily GH replacement therapy alone. Furthermore, the present study indicated that weekly GH replacement therapy with somapacitan could improve glucose intolerance by reducing hepatic insulin resistance compared with daily GH replacement therapy.

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**References**

1. Sassolas G, Chazot FB, Jaquet P, Bachelot I, Chanson P, Rudelli CC, Tauber JP, Allannic H, Bringer J, Roudaut N, Rohmer V, Roger P, Latapie JL, Reville P, Leutenegger M. GH deficiency in adults: an epidemiological approach. Eur J Endocrinol. 1999; 141:595-600.
2. Tritos NA, Biller BMK. Current concepts of the diagnosis of adult growth hormone deficiency. Rev Endocr Metab Disord. 2021; 22:109-116.
3. Nishizawa H, Iguchi G, Murawaki A, *et al.* Nonalcoholic fatty liver disease in adult hypopituitary patients with GH deficiency and the impact of GH replacement therapy. Eur J Endocrinol. 2012; 167:67-74.
4. Molitch ME, Clemmons DR, Malozowski S, Merriam GR, Vance ML; Endocrine Society. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011; 96:1587-1609.
5. White HD, Ahmad AM, Durham BH, Patwala A, Whittingham P, Fraser WD, Vora JP. Growth hormone replacement is important for the restoration of parathyroid hormone sensitivity and improvement in bone metabolism in older adult growth hormone-deficient patients. J Clin Endocrinol Metab. 2005; 90:3371-3380.
6. Ahmad AM, Hopkins MT, Weston PJ, Fraser WD, Vora JP. Effects of GH replacement on 24-h ambulatory blood pressure and its circadian rhythm in adult GH deficiency. Clin Endocrinol (Oxf). 2002; 56:431-437.
7. Crespo I, Santos A, Webb SM. Quality of life in patients with hypopituitarism. Curr Opin Endocrinol Diabetes Obes. 2015; 22:306-312.
8. Ishii H, Shimatsu A, Nishinaga H, Murai O, Chihara K. Assessment of quality of life on 4-year growth hormone therapy in Japanese patients with adult growth hormone deficiency: A post-marketing, multicenter, observational study. Growth Horm IGF Res. 2017; 36:36-43.
9. Ziagaki A, Blaschke D, Haverkamp W, Plöckinger U. Long-term growth hormone (GH) replacement of adult GH deficiency (GHD) benefits the heart. Eur J Endocrinol. 2019; 181:79-91.
10. Pappachan JM, Raskauskiene D, Kutty VR, Clayton RN. Excess mortality associated with hypopituitarism in adults: a meta-analysis of observational studies. J Clin Endocrinol Metab. 2015; 100:1405-1411.
11. Laursen T, Jørgensen JO, Christiansen JS. Pharmacokinetics and metabolic effects of growth hormone injected subcutaneously in growth hormone deficient patients: thigh versus abdomen. Clin Endocrinol (Oxf). 1994; 40:373-378.
12. Jørgensen JO, Flyvbjerg A, Lauritzen T, Alberti KG, Orskov H, Christiansen JS. Dose-response studies with

biosynthetic human growth hormone (GH) in GH-deficient patients. J Clin Endocrinol Metab. 1988; 67:36-40.
13. Abe I, Takeshita K, Nagata M, Fujita Y, Ochi K, Koga M, Kudo T, Shimada H, Abe M, Mukoubara S, Kobayashi K. Investigation of the metabolic and endocrinological differences between daily and weekly growth hormone replacement therapy, somapacitan, in patients with adult growth hormone deficiency: A real-world pilot study. Medicine (Baltimore). 2023; 102:e34730.
14. Ishii K, Abe I, Kameda W, *et al.* Clinical investigation of pituitary incidentalomas: A two-center study. Intractable Rare Dis Res. 2019; 8:239-244.
15. Takeshita K, Abe I, Kameda W, *et al.* Clinical evaluations of pituitary apoplexy in incidental nonfunctional pituitary adenomas. Medicine (Baltimore). 2022; 101:e32026.
16. Otsuka F, Takahashi Y, Tahara S, Ogawa Y, Højby Rasmussen M, Takano K. Similar safety and efficacy in previously treated adults with growth hormone deficiency randomized to once-weekly somapacitan or daily growth hormone. Clin Endocrinol (Oxf). 2020; 93:620-628.
17. Kohno H, Ueyama N, Honda S. Unfavourable impact of growth hormone (GH) discontinuation on body composition and cholesterol profiles after the completion of height growth in GH-deficient young adults. Diabetes Obes Metab. 1999; 1:293-296.
18. Matsumoto R, Fukuoka H, Iguchi G, Nishizawa H, Bando H, Suda K, Takahashi M, Takahashi Y. Long-term effects of growth hormone replacement therapy on liver function in adult patients with growth hormone deficiency. Growth Horm IGF Res. 2014; 24:174-179.
19. Burger AG, Monson JP, Colao AM, Klibanski A. Cardiovascular risk in patients with growth hormone deficiency: effects of growth hormone substitution. Endocr Pract. 2006; 12:682-689.
20. Takahashi Y, Biller BMK, Fukuoka H, Ho KKY, Rasmussen MH, Nedjatian N, Sværke C, Yuen KCJ, Johannsson G. Weekly somapacitan had no adverse effects on glucose metabolism in adults with growth hormone deficiency. Pituitary. 2023; 26:57-72.
21. Johannsson G, Gordon MB, Højby Rasmussen M, Håkonsson IH, Karges W, Sværke C, Tahara S, Takano K, Biller BMK. Once-weekly somapacitan is effective and well tolerated in adults with GH deficiency: a randomized phase 3 trial. J Clin Endocrinol Metab. 2020; 105:e1358-e1376.
22. Johannsson G, Feldt-Rasmussen U, Håkonsson IH, Biering H, Rodien P, Tahara S, Toogood A, Rasmussen MH; REAL 2 Study Group. Safety and convenience of once-weekly somapacitan in adult GH deficiency. a 26-week randomized, controlled trial. Eur J Endocrinol. 2018; 178:491-499.
23. Takahashi Y, Iida K, Takahashi K, *et al.* Growth hormone reverses nonalcoholic steatohepatitis in a patient with adult growth hormone deficiency. Gastroenterology. 2007; 132:938-943.

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# Association of epidural analgesia with childbirth satisfaction among women with severe fear of childbirth: A cross-sectional study

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**SUMMARY**: The relationship between fear severity and satisfaction after vaginal delivery was examined with a focus on its association with epidural analgesia. Postpartum cross-sectional questionnaire survey was conducted among 201 healthy women after vaginal delivery in Japan. The participants completed the Japanese Wijma Delivery Experience Questionnaire (JW-DEQ) Version B and the short version of the Childbirth Experience Self-Rating Scale, and were grouped by use of epidural analgesia and/or fear severity. Data were analyzed using Spearman correlation, Mann–Whitney U, and chi-square tests. No significant association between fear and satisfaction was found in the women without epidural analgesia. The proportion of women with severe fear of childbirth, defined as a JW-DEQ Version B score ≥ 85, was 39%. In the postpartum-recalled severe fear group, epidural analgesia was associated not only with higher overall satisfaction but also higher scores in its sub-factors: "labor pain coping" and "trust in medical staff". In contrast, epidural analgesia was associated only with "labor pain coping" score in the light fear group. Epidural analgesia induced higher childbirth satisfaction among women with severe fear. Although this study is limited by its cross-sectional design, potential recall bias, and the focus on a specific patient population, which may affect the generalizability of the findings, the factors of "labor pain coping" and "trust in medical staff " seem to be important to avoid fear of childbirth for giving childbirth satisfaction.

***Keywords***: Fear of childbirth, epidural analgesia, labor pain, trust

## 1. Introduction

Pregnancy and childbirth are major physical, psychological, and social experiences for many women. Although it can bring great joy to their families, women may experience anxiety and stress as predicting the future course of events is difficult. Hence, they often require medical help, education, social support, and self-help strategies. However, the fear of childbirth among some women exceeds the usual anxiety and leads to tokophobia, a severe fear of pregnancy and childbirth (*1*).

Knauer first described tokophobia in 1897 (*2*). Its prevalence rates in Western countries were over 20% (*3,4*). Meta-analyses and systematic reviews estimated that 14% of pregnant women had tokophobia (*5*). The Wijma Delivery Expectancy/Experience Questionnaire (W-DEQ) (*3*) is an evaluation tool to assess the fear of childbirth. Originally in Swedish, it has now been validated in several other languages and widely used (*6-10*).

Low birth satisfaction among women with a severe fear of childbirth is a pressing issue for healthcare providers. Negative birth experiences can lead to postpartum depression and post-traumatic stress disorder (*11*). The prevalence of postpartum depression was approximately 14% (*12*). Furthermore, 3.2% of women reported post-traumatic stress disorder after childbirth (*13*).

Although vaginal delivery is recommended internationally owing to its advantages, women who suffer from a severe fear of childbirth can request a caesarean section instead of vaginal delivery (*14*). Labor pain, uncertainty of the delivery process, and uncontrollable situations, among other factors, strengthened the fear of childbirth (*15*). The fear of childbirth was also associated with childbirth with epidural analgesia (CwEA) and emergency caesarean section (*16*). Thus, we hypothesized that epidural analgesia would be associated with more positive

childbirth experiences, particularly among women with a severe fear of childbirth. This study aimed to examine the relationship between fear severity and satisfaction after vaginal delivery, with a focus on its association with epidural analgesia across different levels of fear of childbirth.

## 2. Patients and the Method

### 2.1. Patients

This study was conducted at a clinic and hospital in Japan between October and December 2017. A total of 138 and 204 pregnant women visited the clinic and hospital, respectively, for their birth care during the recruitment period. Inclusion criteria were healthy women after vaginal delivery with or without epidural analgesia. Exclusion criteria included women who were younger than 20 years old, could not complete the questionnaire, and underwent a caesarean section.

### 2.2. Research protocol

This study was designed as a postpartum cross-sectional questionnaire survey with prospective recruitment. The study was conducted and reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. Healthy women who had undergone vaginal delivery were recruited by a midwife or nurse at the clinic or hospital. Each participant received a detailed explanation of the study protocol and provided written informed consent. Participants completed self-reported questionnaires, which included the Japanese W-DEQ (JW-DEQ) Version B (*17*), the Short Version of Childbirth Experience Self-Rating Scale (CESRS) (*18*), and a sociodemographic information questionnaire, which included age, height, pre-pregnancy body weight, body weight at delivery, parity, gestational weeks at delivery, and birth weight, among others. They answered the questionnaires and placed them in the collection box in front of the nurses' station prior to discharge. Participants were grouped by epidural use (CwEA vs childbirth without epidural analgesia [Cw/oEA]) and fear severity (JW-DEQ ≥ 85 = severe). To ensure sufficient data for analysis in the smallest subgroup, recruitment was continued until 30 women with CwEA and severe fear of childbirth were enrolled. Since no prior studies had established an effect size for this specific population, a formal power analysis was not conducted, and this study was instead positioned as an exploratory investigation. The total CESRS score was pre-specified as the primary endpoint to assess childbirth satisfaction. The study was reviewed and approved by the Ethics Committee of Kyoto University School of Medicine (No. R1170) in accordance with the Declaration of Helsinki.

### 2.3. Measurements

#### 2.3.1. Japanese Wijma Delivery Experience Questionnaire Version B

The W-DEQ has been widely used to measure the fear of childbirth (*3*). The W-DEQ had two versions: Versions A and B measured expectations regarding fear of childbirth in the antenatal and postnatal period, respectively. Both versions compared fear levels between primiparous and multiparous women (*3*). Each scale comprised 33 items rated on a 6-point scale that ranged from 0 (not at all) to 5 (extremely). The total score ranged from 0 to 165 points. Higher scores indicated a greater degree of fear, with scores over 85 indicating severe fear of childbirth (*3*).

The W-DEQ Versions A and B were translated into Japanese and their validity and reliability were examined among pregnant and postpartum Japanese women (*9,17*). Cronbach's alpha (α) of the JW-DEQ Versions A and B was 0.90 and 0.95, respectively (*9,17*).

#### 2.3.2. Childbirth Experience Self-Rating Scale (Short Version)

The CESRS evaluated childbirth experiences (*19*). This scale comprised four factors and 35 items rated on a 5-point scale that ranged from 1 (not at all) to 5 (extremely). The total score ranged from 35 to 175 points. Cronbach's α of the four factors ranged from 0.91 to 0.80.

Since the 35-item original CESRS was burdensome, Tokiwa created the CESRS (Short Version) (*18*). This scale comprised three factors: "labor pain coping score," "trust in medical staff score," and "childbirth process score." Furthermore, 18 items were rated on a 5-point scale, ranging from 1 (not at all) to 5 (extremely). The total score ranged from 18 to 90 points. Cronbach's α of the three factors ranged from 0.79 to 0.85 (*18*).

### 2.4. Statistical analyses

Spearman's rank correlation coefficient between the JW-DEQ and CESRS was examined (Figure 2). Statistical analyses were performed to compare each fear severity group (Tables 1 and 2) *via* a Mann-Whitney *U*-test and Pearson's chi-square test. Statistical Package Social Sciences (SPSS) version 26.0 (Japan Inc.) was used for all analyses. Two-tailed p-values of $< 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Fear severity and satisfaction during natural vaginal childbirth

Among the 138 and 204 postpartum women in the

clinic and hospital, respectively, 112 (81.2%) and 138 (67.6%) agreed to participate. Hence, 250 postpartum women (73.1%) were included. Of these, 24 who gave birth *via* caesarean section and 25 with missing data were excluded. Finally, data from 201 women were analyzed. We classified participants in the following way: 131 women experienced Cw/oEA and 70 CwEA (Figure 1).

Although a weak correlation tendency was observed between the postpartum-recalled fear (JW-DEQ Version B) and CESRS ($r = -0.203$, $p = 0.020$) among the 131 women who experienced natural vaginal delivery (Cw/oEA) (Figure 2), no statistically significant association was observed. Subsequently, differences in postpartum-recalled fear severity during childbirth were examined across JW-DEQ Version B scores (Table 1). In total, 83 women had < 85 points in the JW-DEQ Version B (light fear group) and 48 women had ≥ 85 points (severe fear group). No statistically significant differences were observed between the light and severe fear groups in terms of median age, height, pre-pregnancy body weight, body weight at delivery, rate of primipara, gestational weeks at delivery, and birth weight. Women in the light fear group had significantly higher total CESRS scores than women in the severe group. Among the sub-factors, "labor pain coping" ($p < 0.001$) and "trust in medical staff " ($p = 0.017$), were significantly different between the groups.

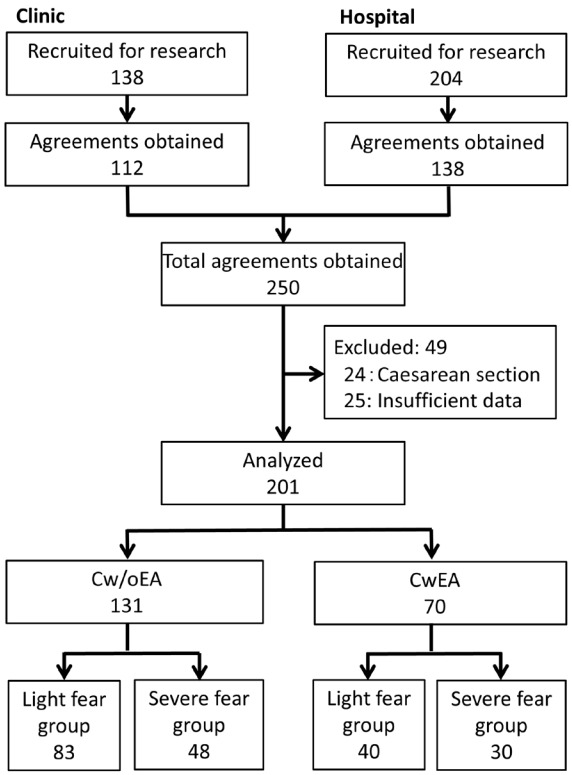


**Figure 1. Flow diagrams.** Abbreviations: Cw/oEA, Childbirth without epidural analgesia; CwEA, Childbirth with epidural analgesia.

### 3.2. Association of epidural analgesia with childbirth satisfaction across fear severity levels

In the light postpartum-recalled fear group, no statistically significant differences were observed in the median age, height, pre-pregnancy body weight, body weight at delivery, rate of primipara, gestational weeks at delivery, and birth weight between the 40 women who chose CwEA and the 83 who did not (Table 2). No statistically significant difference in total CESRS scores was observed between the groups ($p = 0.148$). Only one sub-factor, "labor pain coping," in CwEA was higher than that in Cw/oEA ($p = 0.002$).

In the severe postpartum-recalled fear group, no statistically significant differences were observed in the median height, pre-pregnancy body weight, body weight at delivery, rate of primipara, gestational weeks at delivery, and birth weight between the 30 women who chose CwEA and the 48 women who did not. The age of the women who chose CwEA was significantly higher than those who chose Cw/oEA ($p < 0.001$). The women who chose CwEA had significantly higher total CESRS scores ($p = 0.026$) than those who had Cw/oEA. The two sub-factors of "labor pain coping" ($p < 0.001$) and "trust in medical staff " ($p = 0.024$) were significantly different between the groups.

## 4. Discussion

We demonstrated differences in childbirth experiences associated with epidural analgesia among Japanese women with postpartum-recalled severe fear of childbirth. Among women who chose Cw/oEA, those with severe fear of childbirth had lower CESRS scores than those with light fear. Our results suggest an

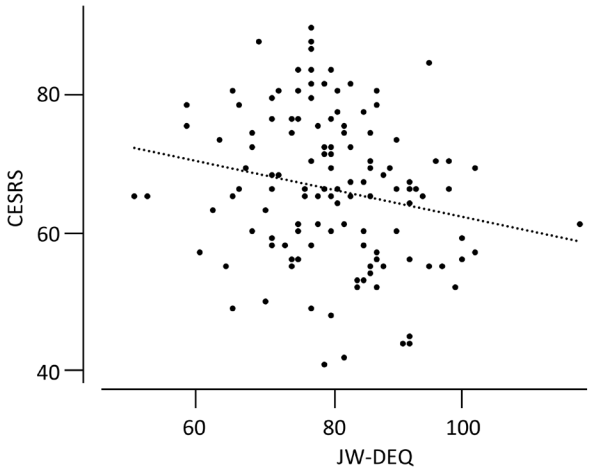


**Figure 2. A correlation between the Japanese W-DEQ (JW-DEQ) and Short Version of Childbirth Experience Self-Rating Scale (CESRS) among the 131 women who experienced natural vaginal delivery ($n = 131$, Spearman's $r = -0.203$, $p = 0.020$).** *Abbreviations*: JW-DEQ, Japanese Wijma Delivery Experience Questionnaire; CESRS, Childbirth Experience Self-Rating Scale.

**Table 1. Characteristics of the women with Cw/oEA**

| Items | Cw/oEA ($n$ = 131) | | |
|---|---|---|---|
| | Light fear group ($n$ = 83) Median (IQR[a]) | Severe fear group ($n$ = 48) Median (IQR[a]) | $P$ value |
| Age (years old) | 31.0 (29.0, 34.5) | 31.0 (28.0, 33.3) | 0.600[b] |
| Height (cm) | 158.0 (155.5, 162.0) | 158.0 (156.0, 162.0) | 0.716[b] |
| Pre-pregnancy body weight (kg) | 49.8 (46.8, 56.4) | 52.0 (46.9, 57.1) | 0.380[b] |
| Body weight at delivery (kg) | 61.9 (56.1, 67.5) | 61.7 (58.0, 68.0) | 0.607[b] |
| Primipara ($n$) | 41 | 27 | 0.377[c] |
| Gestational weeks at delivery (weeks) | 39.0 (39.0, 40.0) | 39.0 (39.0, 40.0) | 0.793[b] |
| Birth weight (g) | 3076 (2912, 3378) | 3155 (2951, 3361) | 0.492[b] |
| JW-DEQ score | 76.0 (72.0, 80.5) | 90.5 (87.0, 95.3) | < 0.001[b] |
| CESRS | | | |
| Total score | 68.0 (62.0, 77.0) | 61.5 (56.0, 70.0) | < 0.001[b] |
| Labor pain coping score | 24.0 (20.0, 27.0) | 19.5 (16.8, 24.0) | < 0.001[b] |
| Trust in medical staff score | 26.0 (23.0, 30.0) | 24.0 (23.0, 27.0) | 0.017[b] |
| Childbirth process score | 20.0 (17.0, 24.0) | 19.5 (16.5, 21.0) | 0.105[b] |

*Abbreviations*: JW-DEQ, Japanese Wijma Delivery Experience Questionnaire; Cw/oEA, Childbirth without epidural analgesia; CESRS, Childbirth Experience Self-Rating Scale. [a]IQR: interquartile range; [b]Mann-Whitney-U test; [c]Pearson's chi-square test.

**Table 2. Characteristics of women in each severity group divided by JW-DEQ score**

| Items | Light Fear Group ($n$ = 123) | | | Severe Fear Group ($n$ = 78) | | |
|---|---|---|---|---|---|---|
| | Cw/oEA ($n$ = 83) Median (IQR[a]) | CwEA ($n$ = 40) Median (IQR[a]) | $P$ value | Cw/oEA ($n$ = 48) Median (IQR[a]) | CwEA ($n$ = 30) Median (IQR[a]) | $P$ value |
| Age (years old) | 31.0 (29.0, 34.5) | 32.0 (29.0, 35.0) | 0.455[b] | 31.0 (28.0, 33.3) | 35.5 (32.0, 38.0) | < 0.001[b] |
| Height (cm) | 158.0 (155.5, 162.0) | 158.0 (155.8, 162.0) | 0.649[b] | 158.0 (156.0, 162.0) | 160.0 (155.5, 163.0) | 0.467[b] |
| Pre-pregnancy body weight (kg) | 49.8 (46.8, 56.4) | 52.0 (47.9, 57.3) | 0.304[b] | 52.0 (46.9, 57.1) | 49.3 (46.3, 54.0) | 0.177[b] |
| Body weight at delivery (kg) | 61.9 (56.1, 67.5) | 62.0 (56.8, 68.0) | 0.703[b] | 61.7 (58.0, 68.0) | 61.5 (57.5, 65.0) | 0.746[b] |
| Primipara ($n$) | 41 | 23 | 0.399[c] | 27 | 23 | 0.085[c] |
| Gestational weeks at delivery (weeks) | 39.0 (39.0, 40.0) | 39.0 (39.0, 40.0) | 0.893[b] | 39.0 (39.0, 40.0) | 39.5 (38.3, 40.0) | 0.781[b] |
| Birth weight (g) | 3076 (2912, 3378) | 3040 (2885, 3325) | 0.595[b] | 3155 (2951, 3361) | 3049 (2750, 3270) | 0.101[b] |
| JW-DEQ score | 76.0 (72.0, 80.5) | 74.5 (72.0, 79.0) | 0.226[b] | 90.5 (87.0, 95.3) | 90.5 (87.0, 94.0) | 0.897[b] |
| CESRS | | | | | | |
| Total score | 68.0 (62.0, 77.0) | 72.5 (66.8, 78.0) | 0.148[b] | 61.5 (56.0, 70.0) | 69.5 (59.5, 75.5) | 0.026[b] |
| Labor pain coping score | 24.0 (20.0, 27.0) | 26.0 (24.8, 30.0) | 0.002[b] | 19.5 (16.8, 24.0) | 25.0 (21.3, 27.8) | < 0.001[b] |
| Trust in medical staff score | 26.0 (23.0, 30.0) | 27.0 (24.8, 29.0) | 0.123[b] | 24.0 (23.0, 27.0) | 26.0 (24.0, 28.0) | 0.024[b] |
| Childbirth process score | 20.0 (17.0, 24.0) | 20.0 (16.0, 22.0) | 0.145[b] | 19.5 (16.5, 21.0) | 19.0 (15.0, 21.0) | 0.433[b] |

*Abbreviations*: JW-DEQ, Japanese Wijma Delivery Experience Questionnaire; Cw/oEA, Childbirth without epidural analgesia; CwEA, Childbirth with epidural analgesia; CESRS, Childbirth Experience Self-Rating Scale. [a] IQR: interquartile range; [b]Mann-Whitney-U test; [c]Pearson's chi-square test.

association between CwEA and improved childbirth experiences in this specific population. The CESRS (Short Version) comprises three subfactors: "labor pain coping", "trust in medical staff" and "childbirth process". Significant differences were observed in the "labor pain coping" and "trust in medical staff" scores.

Severe fear of childbirth has been defined using various measures and cutoffs. In this study, participants were divided into two groups based on their JW-DEQ Version B scores, with scores ≥ 85 indicating severe fear of childbirth, because the research was conducted during the postpartum period. It should be noted that scores on Version B may be influenced by the actual birth experience, potentially resulting in recall bias. The JW-DEQ Version B has been reported to have the same factor structure as Version A, including fear, lack of positive anticipation, isolation, and riskiness, in both primiparous and multiparous women (*17*).

In this study, the proportion of women with severe fear of childbirth, defined as a JW-DEQ Version B score ≥ 85, was 39%. Although this figure does not represent the true prevalence in the general population because of the non-epidemiological sampling design, it is notably higher than both the pooled prevalence of 12% reported using the W-DEQ Version A score ≥ 85 (*5*) and the 19% reported in a previous Japanese study using the JW-DEQ Version B score ≥85 (*20*). Three factors may explain these differences in prevalence. First, cultural and geographical backgrounds differ substantially; the 12 studies reviewed by O'Connell *et al.* (*5*) were conducted across 11 European countries and Australia. Further international research using

globally standardized criteria is needed to clarify these cross-cultural differences. Second, methodological differences between W-DEQ Versions A and B should be considered, as Version A assesses antenatal expectations, whereas Version B evaluates postnatal recall. Third, the discrepancy between this study (39%) and the previous Japanese study (19%) (*20*), despite both using JW-DEQ Version B, may be attributable to selective access to childbirth options. According to a 2020 national survey (*33*), CwEA was available at only 505 facilities (26% of all birthing facilities in Japan) and accounted for only 8.6% of all deliveries (*33*). Because 24-hour analgesic services remain limited in Japan, pregnant women with severe fear of childbirth may proactively seek specialized facilities offering these services. This concentration of women with severe fear of childbirth at specific institutions likely explains the notably high prevalence observed in our sample.

The CESRS (Short Version), developed by Tokiwa, is an original scale created in Japan. A previous study reported scores of 65.8 ± 11.1 for primiparous women and 70.2 ± 9.2 for multiparous women (*34*). Unfortunately, the present study did not classify participants according to parity; however, the score range was similar. Because this scale was developed only in Japanese, international comparisons may be difficult. The Birth Satisfaction Scale-Revised (BSS-R) (*35*), developed in the UK to assess women's childbirth experiences, has been translated into several languages, including Italian, Dutch, Czech, and Japanese. Its validity and reliability have been confirmed previously (*36-40*). The BSS-R was adopted by the International Consortium for Health Outcome Measurement (ICHOM) as a standard measure for pregnancy and childbirth outcomes (*41*). Future research should therefore use the Japanese version of the BSS-R.

In the light fear group, although the "labor pain coping" score in the CwEA group was higher than that in the Cw/oEA group, no statistically significant difference was observed in the total CESRS score. In contrast, in the severe fear group, a statistically significant difference was observed in the total CESRS score, with both the "labor pain coping" and "trust in medical staff" scores being higher in the CwEA group than in the Cw/oEA group. Because labor-inducing agents are often used during CwEA, midwives and nurses frequently visit patients, typically every 30 minutes, to monitor fetal heart rate and provide pain relief support. Physical contact during abdominal monitoring may function as an important form of nonverbal communication that alleviates maternal anxiety and stress, complemented by verbal encouragement in the labor, delivery, and recovery rooms. Consequently, the frequent contact with midwives and nurses associated with CwEA protocols may have contributed to the higher "trust in medical staff" scores. Although the cross-sectional design of this study precludes causal inference, these findings suggest that the intensive monitoring and supportive touch inherent in CwEA protocols may be positively associated with the psychological well-being of women with severe fear of childbirth.

This study has several limitations. First, the cross-sectional design precludes definitive conclusions regarding causality. Second, because the JW-DEQ Version B was administered postnatally, the actual birth experience may have influenced the scores. Although previous studies have reported a significant correlation between prenatal (Version A) and postpartum (Version B) assessments, the postpartum-recalled fear measured in this study may partially reflect the actual delivery experience; therefore, the potential for recall bias and reverse causality should be considered. Third, the voluntary nature of participation may have introduced selection bias, as participants may have been more sensitive to pain than the general population. Fourth, our purposive sampling strategy was designed to ensure a minimum subgroup size rather than to provide population-level epidemiological estimates. Therefore, the 39% prevalence observed in this study likely reflects the specific clinical characteristics of our institution, where a 24-hour epidural service may attract women with severe fear of childbirth, and should not be generalized to the broader population. Fifth, a major limitation was the relatively small sample size after stratification, particularly the subgroup of 30 women with both CwEA and severe fear of childbirth. This limited the statistical power of the comparisons, and the findings should therefore be interpreted cautiously as exploratory results. Sixth, the cutoff value of ≥ 85 for severe fear of childbirth was not derived from a Japanese population. Finally, detailed analgesia-related variables, such as labor pain scores assessed using a Visual Analog Scale and the exact timing of analgesia initiation, were not evaluated, although CwEA was clinically initiated upon maternal request during the active phase of labor.

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**References**

1. O'Connell AM, Leahy-Warren P, Khashan SA, Kenny CL. Tocophobia — The new hysteria? Obstet Gynaecol Reprod Med. 2015; 25:175-177.
2. Knauer O. Uber puerperale Psychosen, fur practische

Aerzte. Karger Publishers; 1897.
3. Wijma K, Wijma B, Zar M. Psychometric aspects of the W-DEQ: A new questionnaire for the measurement of fear of childbirth. J Psychosom Obstet Gynaecol. 1998; 19:84-97.
4. Kanellopoulos D, Gourounti K. A systematic review of tocophobia rate before and during the COVID-19 pandemic. Maedica (Bucur). 2023; 18:455-462.
5. O'Connell MA, Leahy-Warren P, Khashan AS, Kenny LC, O'Neill SM. Worldwide prevalence of tocophobia in pregnant women: Systematic review and meta-analysis. Acta Obstet Gynecol Scand. 2017; 96:907-920.
6. Johnson R, Slade P. Does fear of childbirth during pregnancy predict emergency caesarean section? BJOG. 2002; 109:1213-1221.
7. Fenwick J, Gamble J, Nathan E, Bayes S, Hauck Y. Pre- and postpartum levels of childbirth fear and the relationship to birth outcomes in a cohort of Australian women. J Clin Nurs. 2009; 18:667-677.
8. Garthus-Niegel S, Størksen HT, Torgersen L, Von Soest T, Eberhard-Gran M. The Wijma Delivery Expectancy/ Experience Questionnaire: A factor analytic study. J Psychosom Obstet Gynaecol. 2011; 32:160-163.
9. Takegata M, Haruna M, Matsuzaki M, Shiraishi M, Okano T, Severinsson E. Translation and validation of the Japanese version of the Wijma Delivery Expectancy/ Experience Questionnaire version A. Nurs Health Sci. 2013; 15:326-332.
10. Ortega-Cejas CM, Roldán-Merino J, Lluch-Canut T, Bowen E, Torras-Serrano B, Martínez-Momblan MA, Morera-Balaguer J, Biurrun-Garrido A, Pérez-Botella M, Solà-Perez MD, Mestre-Ferrándiz CM, Moreno-Pimentel AG, Farrero-Muñoz S, de la Cueva-Ariza L, de Dios-Álvarez JL. Reliability and validity study of the Spanish adaptation of the "Wijma Delivery Expectancy/ Experience Questionnaire" (W-DEQ-A). PLoS One. 2021; 16:e0248595.
11. Bell AF, Andersson E. The birth experience and women's postnatal depression: A systematic review. Midwifery. 2016; 39:112-123.
12. Liu X, Wang S, Wang G. Prevalence and risk factors of postpartum depression in women: A systematic review and meta-analysis. J Clin Nurs. 2022; 31:2665-2677.
13. Grekin R, O'Hara MW. Prevalence and risk factors of postpartum post-traumatic stress disorder: A meta-analysis. Clin Psychol Rev. 2014; 34:389-401.
14. Demšar K, Svetina M, Verdenik I, Tul N, Blickstein I, Velikonja VG. Tokophobia (fear of childbirth): Prevalence and risk factors. J Perinat Med. 2018; 46:151-154.
15. Eriksson C, Jansson L, Hamberg K. Women's experiences of intense fear related to childbirth investigated in a Swedish qualitative study. Midwifery. 2006; 22:240-248.
16. Laursen M, Johansen C, Hedegaard M. Fear of childbirth and risk for birth complications in nulliparous women in the Danish National Birth Cohort. BJOG. 2009; 116:1350-1355.
17. Takegata M, Haruna M, Matsuzaki M, Shiraishi M, Okano T, Severinsson E. Psychometric evaluation of the Japanese Wijma Delivery Expectancy/Experience Questionnaire Version B. Open J Nurs. 2017; 7:15-27.
18. Tokiwa Y. Factors affecting self-evaluation of experience of delivery-difference of the primipara and multipara. Annals Gunma Univ Grad Sch Health Sci. 2001; 22:29-39.
19. Tokiwa Y, Imazeki S. Design of self-evaluation scale for experience of delivery and study of its reliability and validity. Jpn J Nurs Sci. 2000; 20:1-9.
20. Iizuka Y, Masaoka N, Ohashi K. Women with fear of childbirth perceived large accumulated labor pain in Japan. Open J Nurs. 2018; 8:656-668.
21. Zar M, Wijma K, Wijma B. Relations between anxiety disorders and fear of childbirth during late pregnancy. Clin Psychol Psychother. 2002; 9:122-130.
22. Kjærgaard H, Wijma K, Dykes AK, Alehagen S. Fear of childbirth in obstetrically low-risk nulliparous women in Sweden and Denmark. J Reprod Infant Psychol. 2008; 26:340-350.
23. Nieminen K, Stephansson O, Ryding EL. Women's fear of childbirth and preference for cesarean section — A cross-sectional study at various stages of pregnancy in Sweden. Acta Obstet Gynecol Scand. 2009; 88:807-813.
24. Spice K, Jones SL, Hadjistavropoulos HD, Kowalyk K, Stewart SH. Prenatal fear of childbirth and anxiety sensitivity. J Psychosom Obstet Gynaecol. 2009; 30:168-174.
25. Adams SS, Eberhard-Gran M, Eskild A. Fear of childbirth and duration of labour: A study of 2206 women with intended vaginal delivery. BJOG. 2012; 119:1238-1246.
26. Nordeng H, Hansen C, Garthus-Niegel S, Eberhard-Gran M. Fear of childbirth, mental health, and medication use during pregnancy. Arch Womens Ment Health. 2012; 15:203-209.
27. Sluijs AM, Cleiren MPHD, Scherjon SA, Wijma K. No relationship between fear of childbirth and pregnancy-/ delivery-outcome in a low-risk Dutch pregnancy cohort delivering at home or in hospital. J Psychosom Obstet Gynaecol. 2012; 33:99-105.
28. Storksen HT, Eberhard-Gran M, Garthus-Niegel S, Eskild A. Fear of childbirth; the relation to anxiety and depression. Acta Obstet Gynecol Scand. 2012; 91:237-242.
29. Salomonsson B, Berterö C, Alehagen S. Self-efficacy in pregnant women with severe fear of childbirth. J Obstet Gynecol Neonatal Nurs. 2013; 42:191-202.
30. Lukasse M, Schei B, Ryding EL, Pelal T, Lou LM, Spy V. Prevalence and associated factors of fear of childbirth in six European countries. Sex Reprod Healthc. 2014; 5:99-106.
31. Toohill J, Fenwick J, Gamble J, Creedy DK. Prevalence of childbirth fear in an Australian sample of pregnant women. BMC Pregnancy Childbirth. 2014; 14:275.
32. Aksoy AN, Ozkan H, Gundogdu G. Fear of childbirth in women with normal pregnancy evolution. Clin Exp Obstet Gynecol. 2015; 42:179-183.
33. Japanese Association for Labor Analgesia. The fact of epidural birth in Japan based on the results of the Survey in 2020 by Ministry of Health, Labour and Welfare, 2022. *https://www.jalasite.org/archives/mutsuu/* (accessed February 17, 2026).
34. Sekizuka N, Nakamura H, Shimada K, Tabuchi N, Kameda Y, Sakai A. Relationship between sense of coherence in final stage of pregnancy and postpartum stress reactions. Environ Health Prev Med. 2006; 11:199-205.
35. Hollins Martin CJ, Martin CR. Development and psychometric properties of the birth satisfaction scale-revised (BSS-R). Midwifery. 2014; 30:610-619.

36. Nespoli A, Colciago E, Pedroni S, Perego S, Fumagalli S. The birth satisfaction scale-revised (BSS-R): Process of translation and adaptation in an Italian context. Ann Ist Super Sanita. 2018; 54:340-347.
37. Nespoli A, Colciago E, Fumagalli S, Locatelli A, Hollins Martin CJ, Martin CR. Validation and factor structure of the Italian version of the birth satisfaction scale-revised (BSS-R). J Reprod Infant Psychol. 2021; 39:516-531.
38. Emmens B, Hollins Martin CJ, Martin CR. Translation and validation of the Dutch version of the birth satisfaction scale-revised (BSS-R). J Reprod Infant Psychol. 2023; 41:213-227.
39. Ratislavová K, Hendrych Lorenzová E, Hollins Martin CJ, Martin CR. Translation and validation of the Czech Republic version of the birth satisfaction scale-revised (BSS-R). J Reprod Infant Psychol. 2024; 42:78-94.
40. Tezuka A, Hiroyama N, Suzuki M, Matsuoka M, Martin CJH, Martin CR. Translation and validation of the Japanese version of the Birth Satisfaction Scale-Revised. Jpn J Nurs Sci. 2024; 21:e12569.
41. Nijagal MA, Wissig S, Stowell C, *et al*. Standardized outcome measures for pregnancy and childbirth, an ICHOM proposal. BMC Health Serv Res. 2018; 18:953.

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# Seasonality of psychiatric symptoms in older adults in long-term psychiatric care

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**SUMMARY**: The annual periodicity of psychiatric symptoms in older adults within long-term care remains under-explored. This exploratory, single-center, retrospective pilot study investigated seasonality in agitation/aggression, depressive symptoms, and hallucinations/delusions among 28 older Japanese inpatients (*mean* age 74.1 ± 9.1 years) over a one-year period to inform the chronobiological optimization of pharmacotherapy. Generalized Linear Mixed-effects Models (GLMMs) with trigonometric terms were used to assess periodicity based on daily clinical records. A possible seasonal pattern, with the model-estimated peak in March and nadir in September, was observed for agitation/aggression (Incidence Rate Ratio [IRR] = 1.50, $p = 0.037$) and depressive symptoms (IRR = 1.08, $p = 0.041$). Hallucinations/delusions showed no significant periodicity. These findings suggest that older adults in long-term care retain sensitivity to seasonal environmental changes, with spring representing a potential period of circadian vulnerability. These findings may help identify periods requiring closer clinical monitoring and medication review, although prospective studies are needed before seasonally guided dosage adjustments can be recommended.

***Keywords***: Seasonal pattern, institutionalization, GLMM

## 1. Introduction

In Japan's psychiatric healthcare system, the average length of hospitalization exceeds 200 days (*1*). With an aging inpatient population, prolonged hospitalization of older adult patients in psychiatric wards poses a significant challenge. This extended period of care often leads to deconditioning and a decline in physical function, which can reduce patients' quality of life (QOL) and increase their risk of mortality. Beyond physical decline, temporal fluctuations in psychiatric symptoms present another critical challenge. Extensive research has shown significant annual seasonality in cognitive function among older adults and patients with dementia, an effect comparable to several years of age-related cognitive change (*2*). Seasonality can exacerbate schizophrenia symptoms (*3,4*), mood episodes in bipolar disorder (*5*), and depression (*6*). Strelnik *et al*. (*7*) suggested the need for more complex temporal factors and individualized predictive models beyond simple seasonality. Existing reports have limitations, including inconsistent definitions of seasons. Particularly, the detailed annual periodicity patterns exhibited by specific symptoms, such as agitation/aggression and hallucinations/delusions, in chronic-phase patients in long-term psychiatric care wards, which is the focus of the present study, have yet to be thoroughly clarified.

In Japanese psychiatric long-term care wards, patients often reside for extended periods with standardized schedules for meals, sleep, and activities. This unique controlled setting minimizes lifestyle-related confounders, allowing for a clearer observation of biological seasonal rhythms driven by environmental cues such as photoperiod. Elucidating these patterns in a controlled long-term care setting provides an objective basis for chronobiological optimization of pharmacotherapy. Understanding these seasonal "windows of vulnerability" is valuable for clinicians to identify periods requiring closer clinical monitoring and timely medication review. Anticipating potential symptomatic peaks, such as the observed spring fluctuation, may help healthcare providers optimize patient care and carefully evaluate need for psychotropic interventions. However, prospective studies are warranted before these seasonal patterns can be used to guide proactive dosage adjustments or prevent polypharmacy in this frail older population.

Beyond pharmacotherapy, non-pharmacological approaches also play a vital role; for instance, increasing

physical activity has been shown to potentially reduce the requirement for psychotropic medications (*8*). However, identifying periods of symptomatic stability is essential for the safe and effective implementation of active interventions. Therefore, this exploratory pilot study aimed to quantitatively investigate the presence and detailed patterns of annual periodicity in specific major psychiatric symptoms, namely agitation/aggression, depressive symptoms, and hallucinations/delusions, among older adult inpatients in long-term psychiatric care wards, using daily clinical records. As a single-center, retrospective study, it was specifically designed to generate hypotheses for future large-scale studies. By elucidating these patterns, we aim to provide a foundation for seasonally tailored pharmacological and non-pharmacological care strategies, including proactive psychiatric rehabilitation and active exercise interventions during stable periods (*9-11*). These findings can be useful for patients, healthcare staff, and family members involved in their care.

## 2. Materials and Methods

### 2.1. Study design

An analytical time-series design was used in this exploratory, single-center, retrospective observational pilot study. The study population included patients who were hospitalized in the long-term psychiatric care wards of a psychiatric hospital in Japan between January 1, 2023, and March 31, 2025, for whom 12 consecutive months of observational data were available.

### 2.2. Ethical considerations

This study was conducted in accordance with the principles of the Declaration of Helsinki (as revised in 2013) and approved by the Institutional Review Board (IRB) of a psychiatric hospital in Japan (approval number: 20250302). Informed consent was obtained *via* an opt-out method approved by the IRB. Details of the study were published on the hospital's official website to ensure that participants had the opportunity to decline participation at any time. Additionally, a notice was posted within the hospital informing potential participants of their right to opt out.

### 2.3. Participants

The participants in this study were older adult inpatients in the long-term psychiatric care ward of a psychiatric hospital in Japan. Initially, a pool of potential participants ($n$ = 50) was identified from those who were hospitalized during the data collection period (January 1, 2023, to March 31, 2025) and met the basic eligibility criteria (length of stay of six months or longer, ability to move independently within the ward with or without a wheelchair, and receiving rehabilitation therapy). From this initial pool ($n$ = 50), participants were selected for the present analysis ($n$ = 28) if they met the following inclusion criteria: (1) availability of medical records for the number of days of symptom occurrence in each month over 12 consecutive months corresponding to a calendar year (January to December). This 1-year observation period was necessary for the statistical analysis of the annual periodicity; and (2) a total length of hospitalization of one year or longer, including a 12-month observation period. This 1-year observation period was selected from the overall data collection period (January 1, 2023, to March 31, 2025).

This criterion was highlighted to exclude the influence of the highly variable acute phase of illness and specifically assess the underlying symptom patterns in patients during a chronic, more stable phase. Furthermore, this population represents a significant challenge in Japanese psychiatric long-term care, making the elucidation of their symptom patterns clinically important. The hospital ward consisted entirely of private (single-occupancy) rooms, and all participants resided in these rooms during the observation period. Participants who did not meet the two key inclusion criteria were excluded. For instance, if their 1-year data were not fully available because the observation period commenced mid-year or ward transfer during the observation period. The detailed participant selection process, including the number of individuals excluded at each stage, specific reasons for exclusion, and distribution of observation years, is illustrated in the Strengthening the Reporting of Observational studies in Epidemiology (STROBE)-style flow diagram (Figure 1). Owing to this rigorous selection procedure, the dataset used in this analysis contained no missing data.

### 2.4. Measurements

#### 2.4.1. Baseline characteristics

Baseline patient characteristics were extracted from medical records. These included age, sex, height, body weight, Body Mass Index (BMI), number of antipsychotic drugs administered, total number of prescribed medications, Mini-Mental State Examination (MMSE) score, history of falls, Functional Independence Measure (FIM) scores, duration of hospitalization, and Charlson Comorbidity Index (CCI) score.

#### 2.4.2. Outcomes

The occurrence of key psychiatric symptoms (agitation/aggression, depressive symptoms, and hallucinations/delusions), defined as the number of days per month in which each symptom was present, was recorded for 1-year period. This information was obtained through a systematic chart review of daily nursing and

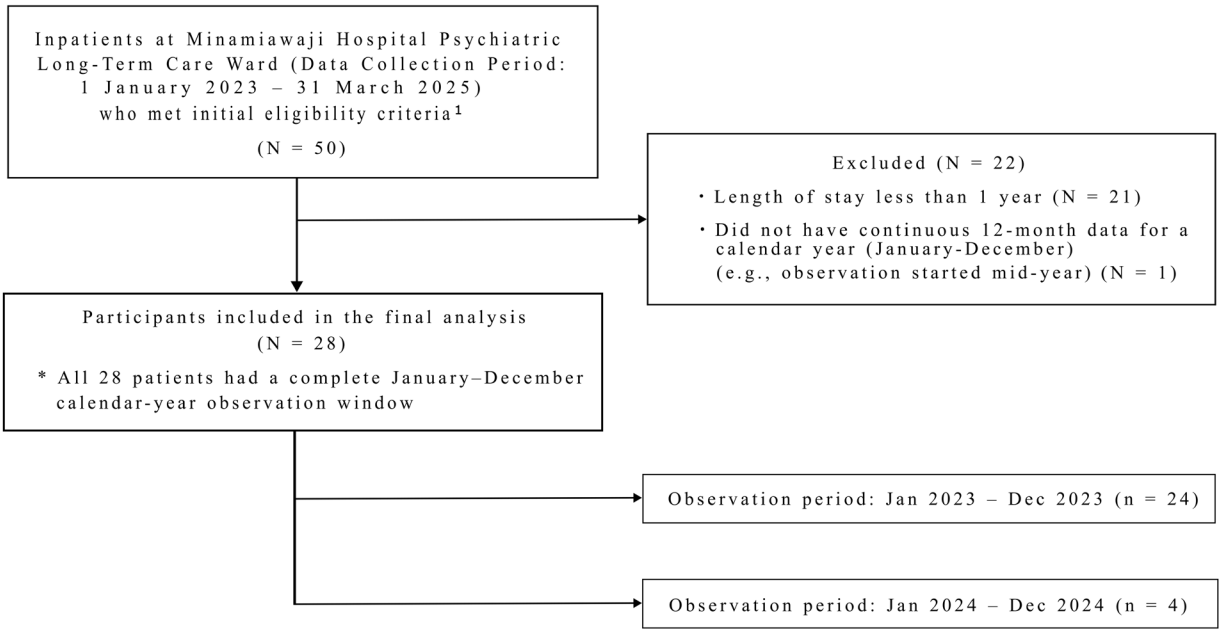


**Figure 1. STROBE-style flow diagram of the participant selection process.**

rehabilitation progress notes by a physical therapist assigned to the ward who possessed longitudinal familiarity with the patients.

2.5. Definition of variables

2.5.1. Agitation/aggression

Agitation/aggression was assessed as a chart-derived indicator based on the Overt Aggression Scale (OAS) concepts (*12*). Specifically, agitation/aggression was considered present if medical records documented behaviors corresponding to the OAS "Verbal Aggression" items (*e.g.*, shouting, loud voice, abusive language, reviling) or "Physical Aggression" items (*e.g.*, hitting, kicking, and throwing objects). The number of days on which symptoms were observed was recorded monthly.

2.5.2. Depressive symptoms

To capture the behavioral manifestations of depression in this long-term care setting, symptoms were operationalized as chart-derived indicators based on the Patient Health Questionnaire-2 (PHQ-2) concepts (*13*). Depressive symptoms were considered present if the medical records indicated observations corresponding to either "little interest or pleasure in doing things" (anhedonia) or "feeling down, depressed, or hopeless" (depressed mood). The number of days on which these behavioral indicators were observed was recorded monthly.

2.5.3. Hallucinations/delusions

The presence of hallucinations or delusions was determined by reviewing medical chart entries for explicit mentions of "hallucinations" or "delusions" or descriptions suggestive of such symptoms (*e.g.*, talking to someone who was not there, seeing, or hearing things that were not actually present). The number of days on which symptoms were observed was recorded monthly.

2.6. Statistical analysis

2.6.1. Descriptive statistics and trend visualization

First, descriptive statistics, including means, standard deviations (*SD*), medians, and ranges, were calculated for patient characteristics (age, sex, and duration of hospitalization) and each outcome variable (monthly number of days of agitation/aggression, hallucinations/delusions, and depressive symptoms). Subsequently, monthly trends for each symptom were plotted at the individual and group average levels to visually examine data patterns.

2.6.2. Temporal dependence analysis

To evaluate the temporal dependence patterns, autocorrelation functions (ACFs) and partial autocorrelation functions (PACFs) were generated for agitation/aggression, depressive symptoms, and hallucinations/delusions. Additionally, the Ljung-Box test was used to assess the presence of overall autocorrelation in each time series.

2.6.3. Assessment of annual periodicity

Annual periodicity was assessed using a trigonometric function model to evaluate seasonality as a continuous

fluctuation pattern with a 1-year cycle rather than defining categorical seasons (*e.g.*, "spring" or "autumn"). The monthly number of days of agitation/aggression, depressive symptoms, and hallucinations/delusions were the dependent variables. Given that these outcome variables represent over-dispersed count data, Generalized Linear Mixed Models (GLMMs) were fitted assuming a negative binomial distribution using the glmmTMB package (version 1.1.7) in R software. The model included fixed effects for the intercept, month (coded 1–12, treated as a continuous variable to control for linear trends), and trigonometric terms (sine and cosine) to capture seasonal patterns. A random intercept for the participant ID was included to account for baseline inter-patient variability. The presence of a seasonal pattern was primarily determined based on the *p*-values from Wald tests for the coefficients of the trigonometric terms ($\beta_{sin}$ for the sine term and $\beta_{cos}$ for the cosine term). If either $\beta_{sin}$ or $\beta_{cos}$ was statistically significant ($p < 0.05$), a seasonal pattern was considered present at the group level. Model fit was assessed using information criteria, including the Akaike information criterion (*AIC*) and Bayesian information criterion (*BIC*), and a simulated residual analysis appropriate for GLMMs. All data were analyzed using R statistical software (version 4.4.2; R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at a two-sided *p*-value of < 0.05.

2.6.4. Power analysis

To address the limitations of the sample size ($n = 28$), a simulation-based power analysis was performed. Informed by effect sizes reported in previous research on seasonal cognitive fluctuations in older adults (*2*) and preliminary trends observed in our dataset (target effect sizes: sine coefficient = 0.6, cosine coefficient = 0.3), we performed a custom simulation using a negative binomial GLMM with a likelihood ratio test (1,000 iterations). The estimated power to detect the specified effect sizes was 100% (95% CI: 0.955–1.000). These results confirmed that the longitudinal design ($T = 12$ observations per participant), despite the modest sample size, provided adequate statistical power to detect the target seasonal patterns.

To address the potential for type I errors and evaluate the robustness of the observed findings, we performed a 2-degree-of-freedom joint likelihood ratio test (LRT) comparing the full model, which included sine and cosine terms, with a nested null model without seasonality terms. Furthermore, because multiple symptom outcomes were analyzed, the *p*-values obtained from the joint tests were adjusted using the Benjamini–Hochberg False Discovery Rate (FDR) method. Finally, sensitivity analyses were conducted by incorporating baseline age and sex as fixed effect covariates within the GLMM framework.

## 3. Results

3.1. Participant characteristics

The final sample for the periodicity analysis consisted of 28 participants (Table 1). The mean age of the participants was 74.1 years (*SD* = 9.1); 11 (39%) were male, and 17 (61%) were female (Table 1). During the observation period, the average number of days per month with agitation/aggression symptoms was 0.6 (*SD* = 1.7), with depressive symptoms was 7.2 (*SD* = 11.6), and with hallucination/delusion symptoms was 0.5 (*SD* = 1.7) (Table 2).

Regarding the baseline medication profile, the mean total number of prescribed medications was 5.3 (*SD* = 2.2). Psychotropic medications included antipsychotics in 17 participants (61%), antidepressants in four participants (14%), and antiepileptics in four participants (14%). As documented in Supplementary Figure S1 (*https://www.ddtjournal.com/supplementaldata/308*), the psychotropic medication burden remained highly stable throughout the 1-year observation window, with more than 85% of the participants maintaining an unchanged regimen across all months. Medication changes observed during spring symptom peaks were predominantly reactive adjustments (dose enhancements) rather than preemptive dose reductions.

3.2. Descriptive statistics and monthly symptom trends

The descriptive statistics for the mean monthly number of days for each major psychiatric symptom are presented in Table 2. The group-average monthly trends for days with agitation/aggression and depressive symptoms visually suggested a pattern of increase in spring and decrease in autumn (Figures 2 and 3). However, considerable variability was observed in the individual trajectories. Figure 4 shows the monthly progression of hallucinations and delusions. For these symptoms, a clear annual pattern at the group-average level was less evident.

3.3. Exploratory analysis of temporal structure

Exploratory analyses, including the ACF, partial autocorrelation function (PACF), and Ljung-Box tests, were performed on group-average monthly data for each symptom. At the group-average level, these analyses did not reveal a clear significant autocorrelation within a 6-month lag (agitation/aggression: Ljung-Box $Q(6) = 6.30$, $p = 0.391$; depressive symptoms: Ljung-Box $Q(6) = 5.22$, $p = 0.516$; hallucinations/delusions: Ljung-Box $Q(6) = 7.63$, $p = 0.266$). However, considering that this finding might have been influenced by the averaging of individual data, a more detailed periodicity analysis was conducted using GLMMs that can analyze individual-level data.

**Table 1. Baseline characteristics of subjects (*n* = 28)**

| Characteristic | *N* = 28 |
|---|---|
| Age (y) | 74.1 (9.1) [72.5 (68.0–80.5)] |
| Sex | |
| female | 17 (61%) |
| male | 11 (39%) |
| Height (m) | 1.5 (0.1) [1.6 (1.5–1.6)] |
| Weight (kg) | 50.3 (11.2) [50.5 (40.9–55.1)] |
| BMI (kg/m²) | 21.0 (3.7) [21.0 (18.0–24.9)] |
| Length of stay (d) | 1158.0 (1476.2) [867.0 (192.8–1638.5)] |
| MMSE | 17.1 (7.9) [18.5 (14.2–22.2)] |
| Stroke | 8 (29%) |
| Hypertension | 7 (25%) |
| Diabetes mellitus | 9 (32%) |
| Dyslipidemia | 4 (14%) |
| Cancer | 6 (21%) |
| Pulmonary disease / Respiratory disease | 5 (18%) |
| Cardiac disease / Heart disease | 7 (25%) |
| Osteoarthritis | 3 (11%) |
| CI score (Charlson Comorbidity Index score) | 6.4 (2.5) [6.0 (5.0–7.5)] |
| GAF score (Global Assessment of Functioning score) | 31.0 (7.1) [30.0 (27.5–35.0)] |
| Mobility status (Ambulatory, Wheelchair-bound) | |
| Independent ambulation | 5 (18%) |
| Wheelchair | 18 (64%) |
| Rollator | 3 (11%) |
| cane | 2 (7.1%) |
| Mental illness diagnosis | |
| Depressive disorder | 2 (7.1%) |
| Schizophrenia | 9 (32.1%) |
| Organic mental disorder | 3 (10.7%) |
| Alcohol dependence | 3 (10.7%) |
| Late-life psychosis | 4 (14.3%) |
| Others[a] | 7 (25.0%) |
| Total number of prescribed medications | 5.3 (2.2) [5.0 (4.0–7.0)] |
| Patients using antipsychotics | 17 (61%) |
| Patients using antidepressants | 4 (14%) |
| Patients using antiepileptics | 4 (14%) |
| Falls in the past year | 15 (53%) |
| Baseline FIM-Motor score (Functional Independence Measure - Motor) | 63.9 (23.3) [69.0 (48.0–84.0)] |
| Baseline FIM-Cognitive score (Functional Independence Measure - Cognitive) | 23.5 (6.5) [24.5 (19.0–27.5)] |
| Baseline FIM-Total score (Functional Independence Measure - Total) | 87.4 (28.5) [96.5 (67.0–110.0)] |

ND: Mean (*SD*) [Median (Q1-Q3)]; *n* (%). [a]The 'Others' category includes one patient each with the following: delusional disorder, somatic symptom disorder, symptomatic psychosis, emotionally unstable personality disorder, delirium, late-life psychosis, and Korsakoff's syndrome.

**Table 2. Descriptive statistics for monthly symptom days**

| Characteristic | *n* = 336 |
|---|---|
| Agitation/Aggression Symptoms | 0.6 (1.7) [0.0 (0.0, 0.0)] (0–17) |
| Depressive Symptoms | 7.2 (11.6) [0.0 (0.0, 9.0)] (0–31) |
| Hallucination/Delusion Symptoms | 0.5 (1.7) [0.0 (0.0, 0.0)] (0–9) |

ND: Mean (*SD*) [Median (Q1, Q3)] (Min - Max).

When the overall seasonal effect was evaluated using the 2-degree-of-freedom joint LRT, the periodic trends for agitation or aggression (joint $\chi^2$= 3.74, *df* = 2, raw *p* = 0.154) and depressive symptoms (joint $\chi^2$ = 4.80, *df* = 2, raw *p* = 0.091) did not reach statistical significance. After the FDR multiple-comparison correction was applied, the adjusted *p*-values were 0.231 for both the symptoms. Sensitivity analyses adjusting for age and sex yielded consistent directional patterns, although statistical significance was similarly attenuated, except for the individual cosine term in the depressive symptom model, which remained significant ($\beta$ = 0.077, *p* = 0.043). Although statistical significance was not maintained after these rigorous adjustments, the individual trigonometric terms initially demonstrated an exploratory signal (Table 3), suggesting a potentially clinically meaningful seasonal trend characterized by a spring peak in this exploratory pilot study.

### 3.4. GLMM analysis of seasonal pattern

To evaluate the seasonal pattern of each psychiatric symptom (agitation/aggression, hallucinations/delusions, and depression), negative binomial GLMMs were fitted using 1 year of monthly symptom-day data from 28 patients admitted to a psychiatric long-term care ward. These models included participant ID as a random

intercept and trigonometric terms (sine and cosine) representing seasonal patterns as a fixed effect. The key results for the fixed effects of these models, which summarize the findings for the three symptoms, are shown in Table 3.

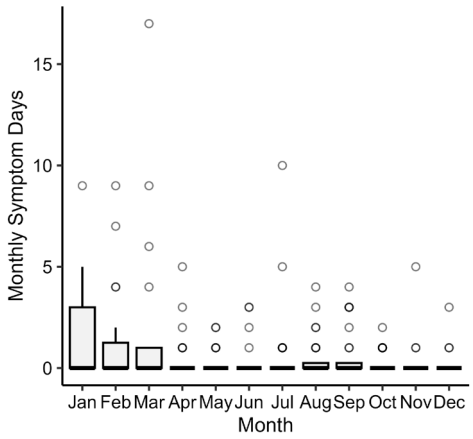


**Figure 2. Monthly box plots of agitation/aggression symptom days.** The box represents the interquartile range (IQR, 25th–75th percentiles) and the line within the box indicates the median. Whiskers extended to the minimum and maximum values within 1.5 × IQR from the box edges. Outliers are denoted by filled circles.

### 3.5. Agitation/aggression symptoms

A possible seasonal trend was observed in the number of days with agitation/aggression symptoms (Table 3). Specifically, the sine term was significant (Incidence Rate Ratio [IRR] = 1.50, 95% confidence interval [CI] 1.02–2.20, $p = 0.037$), whereas the cosine term was not (IRR = 1.18, 95% CI 0.79–1.77, $p = 0.414$). This IRR of 1.50 indicates that the rate of agitation/aggression symptom days increased by 50% at the peak of the cycle compared to the baseline. While a visual inspection of the monthly box plots (Figure 2) might suggest that symptoms are most prominent in January, the GLMM, which accounts for inter-individual variability and intra-individual correlations, identified an underlying periodic pattern in the data. The model estimated that this pattern peaked in spring (March) and reached its nadir in autumn (September) (Figure 5). The amplitude of this seasonal variation (on a log-incidence rate scale) was estimated to be approximately 0.439. Considerable inter-patient variability in baseline symptom levels was observed (variance in random intercept for ID = 5.023). The

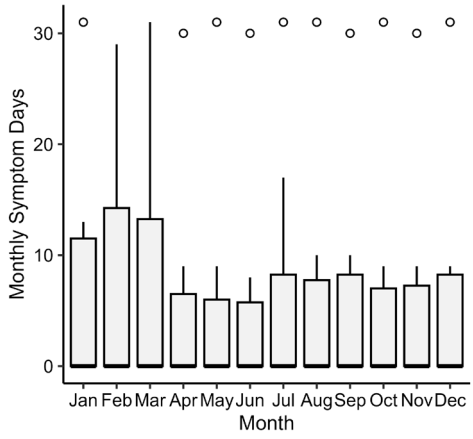


**Figure 3. Monthly box plots of depressive symptom days.** The box represents the interquartile range (IQR, 25th–75th percentiles) and the line within the box indicates the median. Whiskers extended to the minimum and maximum values within 1.5 × IQR from the box edges. Outliers are denoted by filled circles.

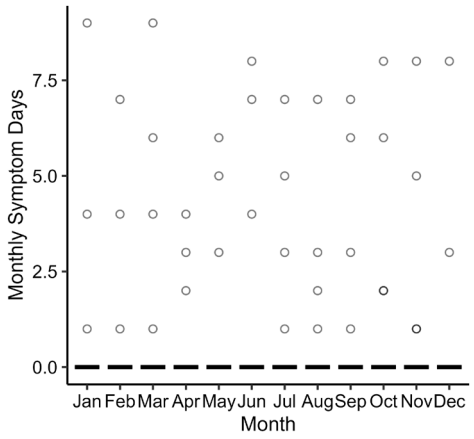


**Figure 4. Monthly box plots of hallucination/delusion symptom days.** The box represents the interquartile range (IQR, 25th–75th percentiles) and the line within the box indicates the median. Whiskers extended to the minimum and maximum values within 1.5 × IQR from the box edges. Outliers are denoted by filled circles.

**Table 3. Generalized Linear Mixed Model (GLMM) analysis of seasonal patterns for psychiatric symptoms ($n = 28$)**

| Symptom | Predictor Variable | IRR | 95% CI | *p*-value |
|---|---|---|---|---|
| Agitation/Aggression Symptoms | sine | 1.50 | 1.02–2.20 | 0.037 |
| | cosine | 1.18 | 0.79–1.77 | 0.414 |
| Depressive Symptoms | sine | 1.08 | 1.00–1.15 | 0.041 |
| | cosine | 1.05 | 0.98–1.12 | 0.177 |
| Hallucination/Delusion Symptoms | sine | 1.11 | 0.67–1.81 | 0.692 |
| | cosine | 1.02 | 0.63–1.65 | 0.939 |

ND: CI = Confidence Interval; IRR = incidence rate ratio. The *p*-values presented here are unadjusted values for the individual trigonometric terms reflecting initial exploratory signals. For the results of the 2-degree-of-freedom joint LRTs, multiple-comparison corrections using the FDR method (adjusted $p = 0.231$ for both agitation/aggression and depressive symptoms), and sensitivity analyses, please refer to the Results section.

model accounted for overdispersion in the data (negative binomial dispersion parameter θ = 0.530). The goodness-of-fit statistics for the model were *AIC* = 548.85 and *BIC* = 571.75.

### 3.6. Depressive symptoms

A possible seasonal trend was also identified in the number of days with depressive symptoms (Table 3). The sine term was significant (IRR = 1.08, 95% CI 1.00–1.15, $p$ = 0.041), whereas the cosine term was not (IRR = 1.05, 95% CI 0.98–1.12, $p$ = 0.177). This corresponds to an estimated 8% increase in the rate of depressive symptom days during the peak period. As with agitation/aggression, although the monthly box plots (Figure 3) show a median value peaking in February, the GLMM, which models the entire continuous annual cycle, revealed a statistically significant periodicity. The estimated periodic pattern showed a slight increase in spring (March) and a slight decrease in autumn (September) (Figure 6). The estimated fixed effect for the sine term, which represents the magnitude of this periodic variation (on a log-incidence rate scale), was 0.072. This magnitude was smaller than that observed for agitation and aggression symptoms (Figure 6). An exceptionally large inter-patient variability in baseline symptom levels was evident (variance of the random intercept for the ID = 91.857). The model accounted for overdispersion in the data (negative binomial dispersion parameter θ = 75.480). The model fit was confirmed with an *AIC* of 1001.35 and a *BIC* of 1024.25.

### 3.7. Hallucination/delusion symptoms

In contrast, no statistically significant seasonal pattern was detected in the number of days with hallucination or delusion symptoms (Table 3, Figure 7). Neither the sine ($p$ = 0.692) nor cosine ($p$ = 0.939) terms were statistically significant. An exceptionally large inter-patient variability in baseline symptom levels was evident (variance in the random intercept for ID = 78.370). The model accounted for overdispersion in the data (negative binomial dispersion parameter θ = 0.796). The goodness-of-fit statistics were *AIC* = 328.06 and *BIC* = 350.97.

## 4. Discussion

In this study, we assessed the seasonal pattern of key psychiatric symptoms using 1-year data on the monthly symptomatic days of 28 patients in a long-term psychiatric care setting in Japan. Although rigorous

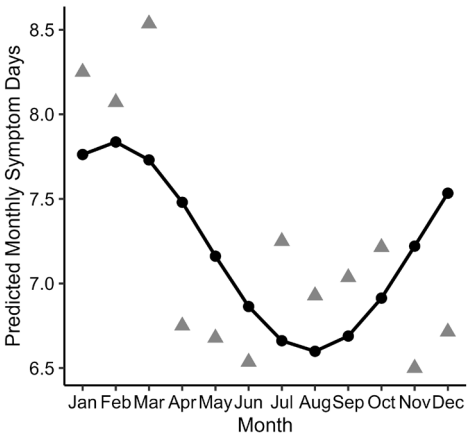


**Figure 6. Predicted annual periodic pattern for depressive symptoms (from GLMM).** The lines and black dots represent the model-predicted monthly expected number of symptom days (group average) from GLMM. The grey triangles indicate the observed mean monthly number of days with symptoms (for reference).

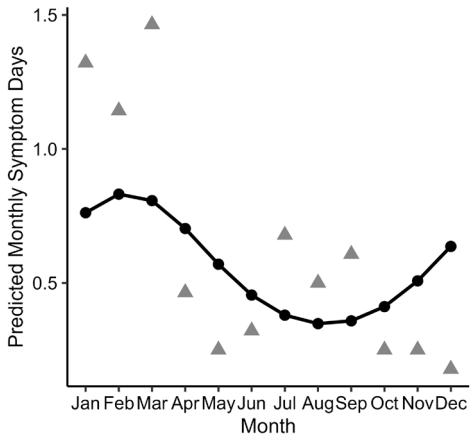


**Figure 5. Predicted annual periodic pattern for Agitation/Aggression symptoms (from GLMM).** The lines and black dots represent the model-predicted monthly expected number of symptom days (group average) from GLMM. The grey triangles indicate the observed mean monthly number of days with symptoms (for reference).

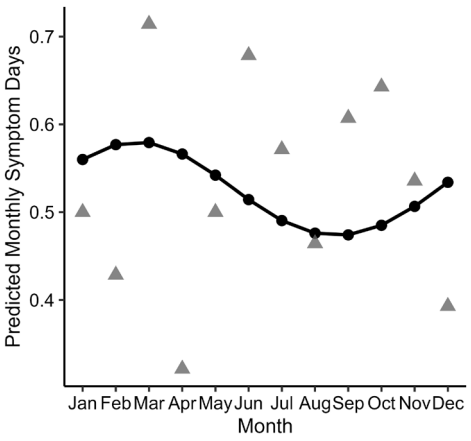


**Figure 7. Predicted annual periodic pattern for hallucination/delusion symptoms (from GLMM).** The lines and black dots represent the model-predicted monthly expected number of symptom days (group average) from GLMM. The grey triangles indicate the observed mean monthly number of days with symptoms (for reference).

joint testing and multiple-comparison corrections reduced statistical significance, exploratory analyses using GLMMs suggested a possible seasonal trend in agitation/aggression and depressive symptoms. These two symptoms shared an annual pattern, with the number of symptomatic days increasing in spring (March–April) and decreasing in autumn (October–November). While these results have highlighted the seasonality of depressive symptoms in individuals with chronic mental illness, they also indicate periodicity in agitation/aggression symptoms, which have previously received limited attention regarding their seasonal patterns.

Although prolonged psychiatric hospitalization in Japan is a unique systemic characteristic, it provides a highly controlled longitudinal environment that is ideal for chronobiological research. Unlike community-dwelling outpatients, the participants in this study followed standardized schedules for light exposure, meals, and sleep, effectively minimizing lifestyle-related confounders. Consequently, while our findings offer preliminary insights into the possible seasonal patterns of older adults in a highly controlled environment because this was an exploratory, single-center, retrospective study, the findings should be interpreted cautiously, and overgeneralization to all long-term care facilities should be avoided. Further multi-center studies are required to determine whether these patterns apply to other facilities with varying environmental structures.

Previous research on seasonal depression has shown mixed patterns. While winter depression in older adults has been attributed to insufficient light exposure, our finding of spring-symptom peaks suggests a different mechanism. This finding aligns with the observations of Sato *et al*. (*14*), who reported that spring can trigger depressive mixed states distinct from classic winter depression. A key factor is the phase advance in the circadian rhythm of older adults (*15,16*). In spring, the timing of dawn advances rapidly. For individuals with an internal clock that is already running early, this accelerated advance of morning light can act as a powerful yet disruptive stimulus, potentially leading to instability in emotional regulation. Furthermore, this vulnerability may be amplified by the diminished amplitude of the circadian signal common in aging (*15,16*). While Lim *et al*. (*2*) found that cognitive function in older adults peaked in late summer and early autumn, our findings suggest that psychiatric symptoms follow a different trajectory, peaking in spring. The interaction between a phase-advanced, low-amplitude circadian system and the dynamic light environment in spring provides a plausible neurobiological mechanism for the observed peaks. This hypothesis is supported by recent sensor-based research demonstrating that environmental triggers directly precede agitation episodes (*17*).

Beyond biological mechanisms, we must consider psychosocial factors specific to long-term care environments. As is common in Japanese healthcare institutions, personnel rotation typically occurs in April. It could be argued that this social stressor solely explains spring exacerbation. However, our analysis suggests a more complex mechanism. While staff rotations occurred, the scale was minimal, and the symptom upward trend began as early as February–March. We propose that the spring peak likely results from a "synergistic interaction" between biological vulnerability (circadian mismatch) (*18,19*) and psychosocial stressors. This "double hit" of biological and social cues renders spring a period of heightened vulnerability.

Regarding agitation/aggression symptoms, for which periodicity was observed in this study, direct reports on their seasonality are limited. Although the involvement of serotonin has been suggested (*20*), this relationship remains unclear (*21*). In contrast, this study did not find any significant seasonal patterns in hallucinations or delusions. Given that the mean monthly occurrence of hallucinations/delusions was nearly identical to that of agitation/aggression, this null finding is highly informative. This highlights the specificity of seasonal effects, suggesting that psychotic symptoms are more strongly influenced by individual pathology or internal neurobiological factors rather than environmental cues such as seasons.

The clinical significance of the observed seasonal fluctuations warrants careful consideration. Although the identified 8% increase in the number of days with depressive symptoms (IRR = 1.08) might appear numerically modest, its impact on a frail population is substantial. Given that depressive symptoms are highlighted as predictors of falls (*22*), this fluctuation translates to an extended "window of vulnerability" during spring (March–April). Furthermore, the 50% increase in agitation/aggression days (IRR = 1.50) represented a more overt and immediate risk to patient safety. For older adults living on the threshold of physical frailty (*23-25*), even a marginal elevation in the symptom baseline can act as a "tipping point," pushing them from a state of precarious balance to functional decline or behavioral incidents. Therefore, the spring peak should not be dismissed as statistical noise but recognized as a critical period where the safety margin is significantly eroded. To address this, care strategies must be proactively adjusted, for instance, by increasing the frequency of behavioral monitoring from standard routines to more intensive checks during March and April. Such targeted, seasonally tailored interventions not only enhance patient safety but also offer a cost-effective approach by preventing severe incidents that necessitate resource-intensive medical care. Furthermore, clinicians must remain vigilant, as these spring behavioral exacerbations often align with other seasonal health risks common in older adults, such as cardiovascular events, potentially compounding the overall clinical management challenges.

Several limitations warrant consideration. First, because this was an exploratory, single-center, retrospective pilot study, the sample size ($n$ = 28) was modest, thereby limiting the generalizability of the findings to other long-term care settings. However, our simulation-based power analysis (estimated at 100%) confirmed that the dense repeated-measures design (12 observations per participant) provided adequate statistical power to detect the target seasonal pattern. Second, the 1-year observation period limits our ability to confirm the multi-year reproducibility of these cycles; further longitudinal studies are required to establish the stability of seasonal patterns. Third, the reliance on daily nursing and rehabilitation records, while offering high ecological validity, lacks the precision of prospective structured diagnostic interviews, and inter-rater reliability was not formally assessed. Furthermore, we could not determine whether the documented symptoms represented new onset, exacerbation of existing symptoms, or improved detection, as nursing documentation practices may have varied during the observation period. Finally, while we accounted for major confounders such as medication adjustments and infectious outbreaks, other unmeasured environmental or psychosocial factors inherent to long-term care may have influenced our findings. Despite these constraints, this study provides critical preliminary evidence for the chronobiological management of psychiatric symptoms in older adults in Japan.

These findings offer an objective rationale for tailoring interventions based on seasonal risks. The risk of worsening spring symptoms could prompt healthcare teams to adjust their staffing or pre-emptively review care strategies. Specifically, agitation and aggression increase the risk of hazardous actions (*26*), whereas significant depressive symptoms increase fall likelihood (*22*). Therefore, we recommend tailoring interventions based on symptomatic states: facilitating de-escalation through non-activating engagement during periods of high excitability (spring), and fostering activity-enhancing interactions when negative symptoms are prominent. Moreover, these findings provide vital preliminary information for planning home discharge and community re-entry, as families can be educated about seasonal periods of instability. Clinically, these patterns suggest the need for proactive measures; for instance, increasing the frequency of monitoring or preventive rehabilitation during the high-risk months of March and April could help mitigate these risks. Future research should aim to elucidate how these seasonal variations correlate with physical functioning (activities of daily living, ADL) and QOL to further refine these interventions.

Although preliminary, our findings suggest that in older adult patients with chronic conditions residing in long-term psychiatric care facilities, the symptoms of agitation/aggression and depression tended to peak in spring and were least prevalent in autumn. These findings underscore the importance of considering seasonal factors in the development of preventive approaches and individualized care plans for these symptoms.

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**References**

1. Ministry of Health, Labour and Welfare of Japan. Overview of the 2022 Survey of Medical Institutions (Static and Dynamic) and Hospital Report. *https://www.mhlw.go.jp/toukei/saikin/hw/iryosd/22/dl/03byouin04.pdf* (accessed March 28, 2026).
2. Lim ASP, Gaiteri C, Yu L, *et al*. Seasonal plasticity of cognition and related biological measures in adults with and without Alzheimer disease: Analysis of multiple cohorts. PLoS Med. 2018; 15:e1002647.
3. Abe K, Suzuki T, Egashira K. Seasonal patterns of recurrence in schizophreniform psychosis. Schizophr Res. 1992; 7:43-47.
4. Hinterbuchinger B, König D, Gmeiner A, Listabarth S, Fellinger M, Thenius C, Baumgartner JS, Vyssoki S, Waldhoer T, Vyssoki B, Pruckner N. Seasonality in schizophrenia-An analysis of a nationwide registry with 110,735 hospital admissions. Eur Psychiatry. 2020; 63:e55.
5. Geoffroy PA, Bellivier F, Scott J, Etain B. Seasonality and bipolar disorder: A systematic review, from admission rates to seasonality of symptoms. J Affect Disord. 2014; 168:210-223.
6. Øverland S, Woicik W, Sikora L, Whittaker K, Heli H, Skjelkvåle FS, Sivertsen B, Colman I. Seasonality and symptoms of depression: A systematic review of the literature. Epidemiol Psychiatr Sci. 2019; 29:e31.
7. Strelnik S, Strelnik A, Astafeva D, Romanov D. Chronobiological predictors of depression relapse: For prognostic model development—A systematic review. Psychiatr Danub. 2023; 35(Suppl 2):56-65.
8. Hirschbeck A, Leao DS, Wagner E, Hasan A, Roeh A. Psychiatric medication and physical performance parameters – Are there implications for treatment? Front Psychiatry. 2022; 13:985983.
9. Carpels A, de Smet L, Desplenter S, de Hert M. Falls among psychiatric inpatients: A systematic review of literature. Alpha Psychiatry. 2022; 23:217-222.
10. Loh PY, Martinengo L, Heaukulani C, Tan XY, Hng

M, Cheah YY, Morris RJT, Tudor Car L, Lee J. Characteristics and outcomes of mHealth interventions in psychosis: Systematic mapping review. J Med Internet Res. 2024; 26:e55924.
11. Tang L, Zhang L, Liu Y, Li Y, Yang L, Zou M, Yang H, Zhu L, Du R, Shen Y, Li H, Yang Y, Li Z. Optimal dose and type of exercise to improve depressive symptoms in older adults: A systematic review and network meta-analysis. BMC Geriatr. 2024; 24:505.
12. Yudofsky SC, Silver JM, Jackson W, Endicott J, Williams D. The Overt Aggression Scale for the objective rating of verbal and physical aggression. Am J Psychiatry. 1986; 143:35-39.
13. Arroll B, Goodyear-Smith F, Crengle S, Gunn J, Kerse N, Fishman T, Falloon K, Hatcher S. Validation of PHQ-2 and PHQ-9 to screen for major depression in the primary care population. Ann Fam Med. 2010; 8:348-353.
14. Sato T, Bottlender R, Sievers M, Möller HJ. Distinct seasonality of depressive episodes differentiates unipolar depressive patients with and without depressive mixed states. J Affect Disord. 2006; 90:1-5.
15. Duffy JF, Zeitzer JM, Rimmer DW, Klerman EB, Dijk DJ, Czeisler CA. Peak of circadian melatonin rhythm occurs later within the sleep of older subjects. Am J Physiol Endocrinol Metab. 2002; 282:E297-E303.
16. Scholtens RM, van Munster BC, van Kempen MF, de Rooij SE. Physiological melatonin levels in healthy older people: A systematic review. J Psychosom Res. 2016; 86:20-27.
17. Davidoff H, van Kraaij AV, Lutin E, van den Bulcke L, Vandenbulcke M, van Helleputte N, de Vos M, van Hoof C, van den Bossche M. Environmental triggers of specific subtypes of agitation in people with dementia: Observational study. JMIR Form Res. 2025; 9:e60274.
18. Srinivasan V, Smits M, Spence W, Lowe AD, Kayumov L, Pandi-Perumal SR, Parry B, Cardinali DP. Melatonin in mood disorders. World J Biol Psychiatry. 2006; 7:138-151.
19. Zhang R, Volkow ND. Seasonality of brain function: Role in psychiatric disorders. Transl Psychiatry. 2023; 13:65.
20. Krämer UM, Riba J, Richter S, Münte TF. An fMRI study on the role of serotonin in reactive aggression. PLoS One. 2011; 6:e27668.
21. Duke AA, Bègue L, Bell R, Eisenlohr-Moul T. Revisiting the serotonin-aggression relation in humans: A meta-analysis. Psychol Bull. 2013; 139:1148-1172.
22. Feng Z, Chen Q, Li Y, Xue Z, Hao X. The association between falls and depressive symptoms amongst older adults: Evidence from the China Health and Retirement Longitudinal Study. Front Public Health. 2023; 11:1248551.
23. Chen YZ, Huang ST, Wen YW, Chen LK, Hsiao FY. Combined effects of frailty and polypharmacy on health outcomes in older adults: Frailty outweighs polypharmacy. J Am Med Dir Assoc. 2021; 22:606.e7-606.e18.
24. Hajek A, Brettschneider C, Posselt T, *et al*. Predictors of frailty in old age - results of a longitudinal study. J Nutr Health Aging. 2016; 20:952-957.
25. Sousa CR, Coutinho JFV, Freire Neto JB, Barbosa RGB, Marques MB, Diniz JL. Factors associated with vulnerability and fragility in the elderly: A cross-sectional study. Rev Bras Enferm. 2022; 75:e20200399.
26. Kim SC, Kaiser J, Bulson J, Hosford T, Nurski A, Sadat C, Kalinowski N. Multisite study of Aggressive Behavior Risk Assessment Tool in emergency departments. J Am Coll Emerg Physicians Open. 2022; 3:e12693.

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***Original Article***

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# Exploratory study on the effect of ferulic acid derived from rice bran on dull skin

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**SUMMARY**: Ferulic acid (FA) from rice bran is known to possess strong antioxidant properties. Furthermore, its multifunctional properties, such as skin whitening, anti-glycation, and anti-inflammatory effects, have attracted attention. We undertook an exploratory study to examine whether FA might be associated with improvements in skin dullness. A single-blind study was conducted on 24 healthy women, in which a cream containing 1% FA was applied to one side of the face and a placebo cream was applied to the other side to verify the effects. To explore the molecular mechanism, RNA-seq was performed on HEKn keratinocyte and Hs68 fibroblast, followed by qRT-PCR. In the human clinical trial, the melanin index significantly decreased in the area where the FA cream was applied, and a questionnaire also subjectively confirmed the improvement of dark and yellowish dullness by FA cream. RNA-seq analysis of HEKn cells treated with FA revealed significant changes in the expression of FGF-2 (basic fibroblast growth factor) promoting melanocyte proliferation, and CD36 (cluster of differentiation 36), related to the removal of advanced glycation end-products (AGEs). qRT-PCR confirmed that FA significantly downregulated FGF-2 and upregulated CD36 expression. These results suggest that FA may exert its effects by regulating the expression of genes involved in melanogenesis and glycation, contributing to the suppression of melanin production and the reduction of AGEs for the improvement of dull skin.

***Keywords***: Dull skin, ferulic acid, glycation, melanin, thickened stratum corneum

## 1. Introduction

Ferulic acid (FA), a phenolic compound is known to act as a scavenger and possesses strong absorption in UV (*1-3*). The structure of FA is shown in Supplementary Figure S1 (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*). With the growing interest in health and beauty, much attention has been paid to the other functions of FA, such as antioxidant, anti-inflammatory, and anti-bacterial activity (*1,4,5*).

Dull skin is one of the most common skin problems among various racial and cultural backgrounds. In addition to intrinsic factors, such as darkening of skin tone due to melanin accumulation and changes in the blood flow, dullness might be related to extrinsic factors, such as reduced light reflectance and roughness of the skin. Furthermore, the visual impression of dullness is exacerbated by the loss of skin transparency with aging (*6,7*).

Advanced glycation end-products (AGEs) accumulated in the skin with aging and amplified by exogenous factors, such as UV, cause dull and yellowish skin (*8*). The Japan Cosmetic Industry Association states that one of the causes of dullness is a decrease in transparency due to thickening of the stratum corneum.

Common treatments for dull skin include arbutus and vitamin C derivatives for whitening and exfoliation treatment such as peeling (*9,10*). Although these methods have shown certain effects, they tend to rely on a single mechanism of action and there are issues, such as variation in effects among individuals and irritation and deterioration of the barrier function of the skin with long-term use (*11*). In this study, we examined the effects of a cream containing 1% FA (FA cream) and a placebo cream in a human clinical trial. Moreover, using human epidermal keratinocytes (HEKn cells) and dermal fibroblast cells (Hs68 cells), the expression levels of genes related to melanin production and glycation were examined by quantitative real-time PCR (qRT-PCR).

## 2. Materials and Methods

### 2.1. Reagents

The FA used in this study was provided by Tsuno Food Industry Co., Ltd. (Wakayama, Japan), and its purity was more than 98% (Lot number: F09883).

### 2.2. Stability tests of FA

Placebo and FA creams were formulated with FA concentrations of 0, 0.5, 1.0, 1.5, and 2.0%. The composition is shown in Supplementary Table S1 (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*). These formulations were stored at 40°C for 30 days. After the storage, the creams were evaluated for discoloration and odor in Supplementary Figure S2 (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*).

### 2.3. Human clinical trial

This clinical trial was approved by the ethics committee of Tsuno Food Industry Co., Ltd. (Rin 24-002) and conducted in accordance with the Declaration of Helsinki. This trial was registered in the UMIN Clinical Trials Registry (UMIN000058687) and performed from fall to winter. Twenty-four healthy Japanese women 30 to 50 years old received a cream containing 1% FA and placebo cream to apply twice a day for 12 weeks. They were distributed to subjects once a month for the three months. Each subject applied FA cream to one side of the face and placebo cream to the other. The number of subjects applying the test product to the right side and the number of subjects applying the product to the left side were assigned equally. Assignments as to which cream was applied to which side were kept confidential until all subjects had completed the study (single-blind study). Prior to the measurement, subjects were acclimatized for 30 min in a constant temperature and humidity chamber (22°C, 50% humidity) after washing their faces. Using a Mexameter MX18 (Courage+Khazaka electronic GmbH, Cologne, Germany), the melanin index was measured on both sides of the face at week 0 and every 4 weeks thereafter, for a total of four measures. We used the VISIA Evolution system (Canfield Scientific, Parsippany, NJ, USA) to photograph subjects' faces. At least three measures were performed on both sides of faces. Imaging was conducted under standardized and reproducible conditions in accordance with the manufacturer's guidelines. Lighting conditions were controlled using the system's built-in cross-polarized and UV illumination modes. Camera settings were kept constant across all measurements. In addition, subject positioning was standardized using chin and forehead stabilizers to ensure consistency with previously analyzed images. We utilized VISIA Evolution system to measure the spots in their faces and analyzed using the included software. Using Corneometer CM825 (Courage+Khazaka electronic), the stratum corneum water content was measured. This was based on the capacitance measurement of a dielectric medium and the hydration level could be detected in this system. At least three measurements were performed on both sides of each subject's face.

We performed the Visual Analog Scale (VAS) to obtain subjective evaluation of skin changes and feeling including touch and appearance after utilization. Subjects were asked to answer a questionnaire using a VAS at week 0 and 12. The degree of skin condition that they felt was recorded on a 10 cm (0 cm = very bad, 10 cm = very good). This allowed participants to express their perception on a continuous scale between two extremes.

In this study, the handling of participants' personal information and the protection of privacy regarding the publication of study results were thoroughly explained during a pre-study briefing. Signed informed consent was obtained from each participant to ensure respect for their privacy. We obtained additional informed consent regarding the publication of the facial images. To protect the participants' privacy, all images were anonymized.

### 2.4. Cell culture

Normal human epidermal keratinocytes isolated from neonatal foreskin (HEKn) were cultured in EpiLife™ Medium containing 10% Human Keratinocyte Growth Supplement (HKGS) (Gibco™, Thermo Fisher Scientific, Waltham, USA), 100 U/mL penicillin and 60 μM $CaCl_2$ at 37°C under 5% $CO_2$. Human dermal fibroblast cells (Hs68) were also cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 10 mg/mL streptomycin (Fujifilm Wako Pure Chemical, Osaka, Japan). HEKn and Hs68 cells were suspended in 24-well plates (WATSON, Tokyo, Japan) at $1 \times 10^5$ and $2 \times 10^5$ (cells/well), respectively, and precultured overnight at 37ºC.

### 2.5. Measurement of cell viability

FA was added to HEKn and Hs68 cells at 0 - 5 mM, followed by incubation for 24 h at 37°C. After treatment, the culture medium was removed and replaced with serum-free DMEM containing 10% tetrazolium salt reagent (cell counting kit-8, Dojindo Laboratories, Kumamoto, Japan). Cells were then incubated for 2 h and cell viability was assessed by the absorbance at 450 nm (Tecan, Kanagawa, Japan). Relative viability was calculated by normalizing to the untreated group.

### 2.6. RNA sequencing (RNA-seq) for profiling of gene expression

HEKn and Hs68 cells were incubated with FA at 0.2 and 1 mM, and 0.4 and 2 mM, respectively, for 24 h at 37°C and total RNA was extracted. A TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA) was utilized to construct the library. For RNA-seq, next-generation sequencing (NGS) was performed using NovaSeq6000 (Illumina).

2.7. Expression levels of melanogenesis-related genes

HEKn cells were incubated with FA (0.5, 1, and 1.5 mM) for 4 h at 37°C and total RNA was obtained. cDNA was synthesized from total RNA (250 ng) using PrimeScript RT reagent (TAKARA BIO, Shiga, Japan). Using TB GreenR Premix Ex Taq™ II, qRT-PCR was performed. Each gene expression level was calculated relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences for GAPDH, basic fibroblast growth factor (FGF-2), hepatocyte growth factor (HGF), stem cell factor (SCF), and neuregulin 1 (NRG1) were designed from the following literature (*12-16*) and shown in Supplementary Table S2 (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*).

2.8. Expression levels of glycation-related genes

Expression levels of CD36 were measured by qRT-PCR, as well as that of melanogenesis-related genes. HEKn cells were incubated with FA (0.5, 1, and 1.5 mM) for 24 h and treated with Isogen II to obtain total RNA. cDNA was obtained using the PrimeScript RT reagent. The primer sequences for CD36 and GAPDH were designed following the cited literature and shown in Supplementary Table S2 (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*) (*12,17*).

2.9. Statistical analysis

Statistical analyses were performed using JMP software (SAS Institute Inc., Cary, NC, USA). A paired *t*-tests were used for the VAS questionnaire, while Dunnett's test was applied to the melanin index measurements, cell viability, and gene expression. All data were shown as means ± SEM. The significance level for each test was set at 5% or less.

**3. Results**

3.1. Changes in color and smell of FA cream

In Supplementary Figure S2 (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*), obvious discoloration and odor change were observed in creams containing more than 1.5% FA compared to placebo cream. Neither odor nor color change were observed for creams containing 0, and 0.5% FA creams, as well as placebo cream. In creams containing 1% FA, a slight change in discoloration was observed, but no change in odor was observed at 40°C. Consequently, 1% FA cream was utilized and new creams were provided to subjects once a month in the human clinical trial.

3.2. Subject grouping in the human clinical trial

Subjects were assigned to two groups to ensure that the mean baseline values of the primary outcome (melanin content) and the secondary outcomes (stratum corneum water content and spot score) were equivalent (Figure 1A and 1C). No significant differences were observed in the baseline values of these parameters between FA-treated and placebo groups. The study design for the topical application of FA was summarized in Figure 2. Twenty-nine subjects were evaluated for eligibility, but as one of them did not meet the selection criteria of spot score, only the remaining 28 subjects were randomized into two groups. One group applied FA cream to the right side of the face, while the other applied FA cream on the left side. During the trial, four subjects withdrew due to personal reasons. Finally, 24 subjects, ranging in age from 30 to 50 years old, with a mean age of 41 years, completed the 12-week treatment.

3.3. Human clinical trial

The primary end-point of the change in the melanin index was shown in Figure 3 as zero before placebo and FA application. In the area where FA cream was continuously applied, the melanin index significantly decreased eight weeks after the start of application, with a change of − 8.6 ± 1.53 compared to week 0. Although there was no statistically significant difference between FA and placebo groups at week 8, the reduction in melanin index in the FA group was approximately 1.35 times greater than in the placebo group.

As shown in Supplementary Figure S4A (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*), the amounts of stratum corneum water content in the placebo group decreased significantly between week 4 and week 12 from −6.11 ± 2.04 to −11.44 ± 1.95 (week 12, $p < 0.0001$). In contrast, in the FA group, the stratum corneum water content decreased significantly between week 8 and week 12 from −10.14 ± 1.82 to −8.75 ± 1.88 (week 8, $p < 0.0002$; week 12, $p = 0.0014$). As shown in Supplementary Figure S4B (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*), spot score in the placebo group and the FA group at week 4 after the application were −1.50 ± 0.71 (week 4, $p = 0.159$) and −1.28 ± 0.90 (week 4, $p = 0.335$), respectively. From week 4 to week 8, the score of the placebo group continued to improve, while that of the FA group increased and returned to pre-application levels. At week 12, the spot scores of the two groups crossed.

The VAS questionnaire survey that was conducted

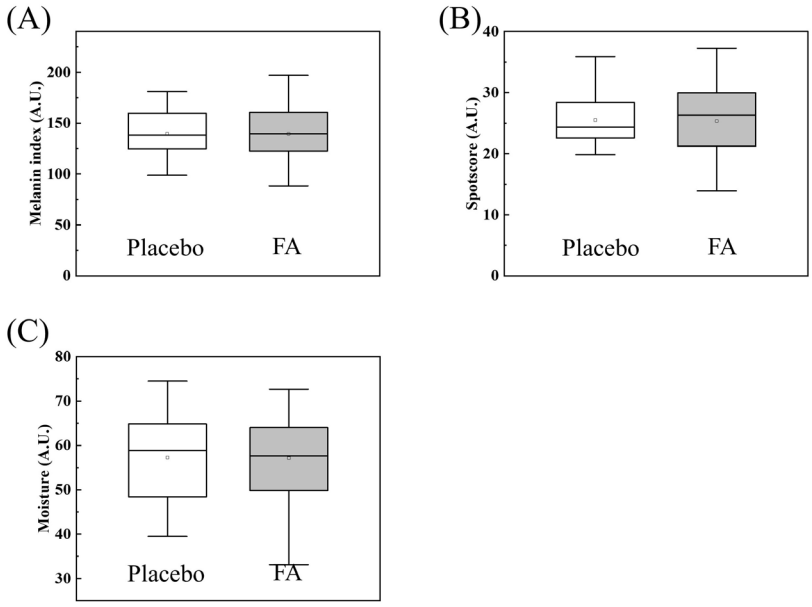


**Figure 1. Box plot.** A box plot for each measurement item after allocation is shown. The white bar shows the values of sides that were treated with placebo cream, while the gray bar shows the values of sides that were treated with FA creams. **(A)** Vertical axis is melanin value (A.U.). **(B)** Vertical axis is spot score (in arbitrary units) relative to number of spots. **(C)** Vertical axis is stratum corneum water content (A.U.).

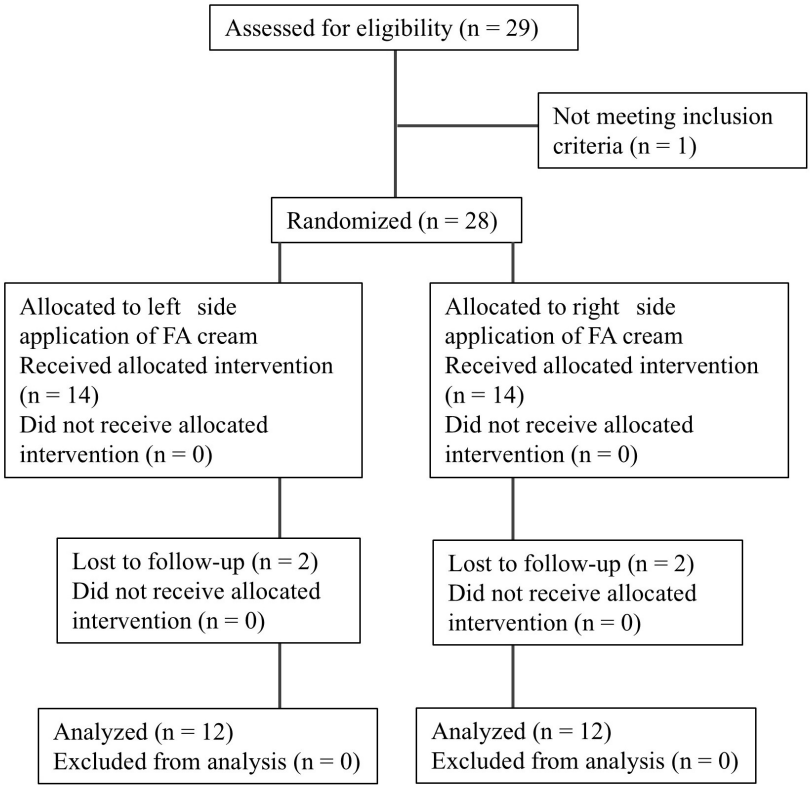


**Figure 2. A total of 29 subjects were assessed for eligibility.** One subject was excluded for not meeting the inclusion criteria based on spot score. The remaining 28 subjects were randomly assigned to two groups: one group ($n$ = 14) applied FA cream to the right side of the face, and the other group ($n$ = 14) to the left side. During the study period, four subjects withdrew due to personal reasons or health-related issues. A total of 24 subjects (mean age: 41 years) completed the 12-week treatment.

as an evaluation of the application experience was performed from week 0 to week 12 and these results of the evaluation items were shown in Figure 4. In the item related to evaluating the brownish dullness caused by melanin production, the FA result was 0.44 ± 0.51 points at week 12, a slight increase compared to that of the placebo group (Figure 4A). In the categories of moisture and dark dullness, the FA group showed significant improvements, with changes of 0.75 ± 0.42 and 0.73 ± 0.50 points, respectively (Figure 4B and 4C). The evaluation item related to yellowish dullness caused by glycation at week 12 in the FA group was observed to change to 1.16 ± 0.49 points (Figure 4D). There were also significant improvements in the overall ratings for skin dullness and skin translucency in the FA group that were also observed at week 12, with changes of 0.78 ± 0.24 and 0.65 ± 0.24 points, respectively (Figure 4E and 4F).

Figure 5A and 5B showed facial images of two subjects at pre- (week 0) and post-application (week 12) taken with the VISIA Evolution. In both subjects, there was an overall improvement in skin lightness compared to pre-application, with a reduction in dullness, particularly in the cheek area.

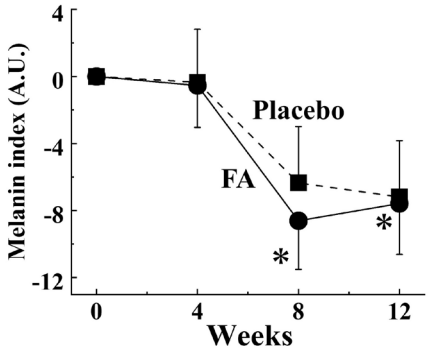


**Figure 3. Effects of FA on dull skin.** The dotted line shows placebo treatment sides, and the straight line shows the FA treatment sides (FA group, $n = 24$; placebo group, $n = 24$). Amount of change of skin melanin index from prior to cream application (week 0) is shown. The vertical axis is the skin melanin index, and the horizontal axis is the number of weeks from the start of the application. Data are shown as mean ± SEM and were analyzed by paired *t*-tests between the FA and placebo groups and by Dunnett's test to compare week 0 in each group. No significant difference was observed between the placebo and FA groups (week 8, $p = 0.320$). In the FA group, significant decreases were observed at week 8 ($p = 0.0149$) and week 12 ($p = 0.0314$) compared to week 0. *$p < 0.05$.

## 3.4. Measurement of cell viability

FA at various concentrations was administered to HEKn and Hs68 cells, and cell viability was assessed. For HEKn cells, FA up to 0.5 mM promoted cell proliferation. In contrast, 5 mM FA significantly reduced cell viability to 42.9%, consistent with cytotoxicity. From

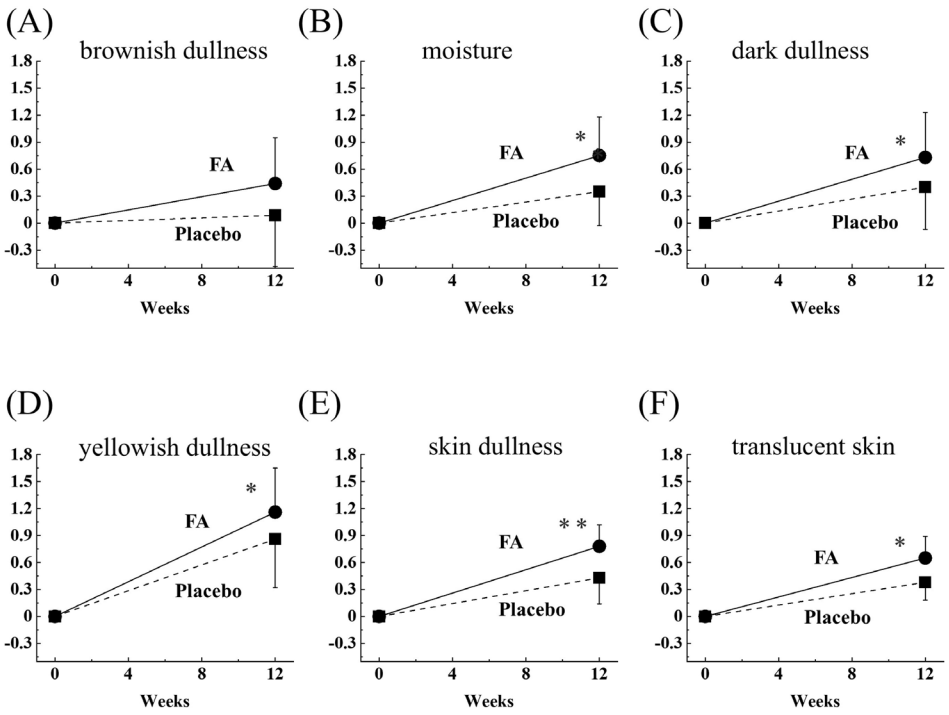


**Figure 4. Self-assessment of skin condition throughout the treatment period. (A)** Subjective improvement in brown dullness. **(B)** Subjective improvement in moisture. **(C)** Subjective improvement in dark dullness. **(D)** Subjective improvement in yellowish dullness. **(E)** Subjective improvement in overall dullness of the skin. **(F)** Subjective improvement in skin translucency. Data are expressed as means ± SEM ($n = 24$). Data were analyzed by paired *t*-tests. *$p < 0.05$, **$p < 0.01$ *vs.* significantly different from FA week 0. AU, arbitrary units. At week 12, paired t-tests were used to compare the placebo group and FA group (brownish dullness, $p = 0.093$; moisture, $p = 0.134$; dark dullness, $p = 0.242$; yellowish dullness, $p = 0.197$; skin dullness, $p = 0.83$; translucent skin, $p = 0.215$).

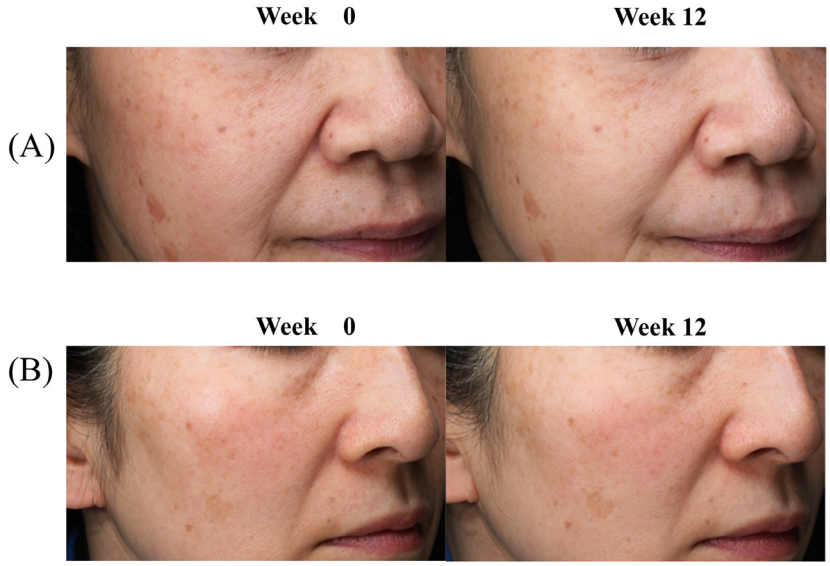


**Figure 5. Facial images of two subjects (A and B) at pre- (week 0) and post-application (week 12), were taken with the VISIA Evolution. (A)** Upper left: before application (week 0); upper right: after 12 weeks of FA cream application. **(B)** Lower left: before application (week 0); lower right: after 12 weeks of FA cream application.

these findings, the upper limit of a safe concentration of FA was determined to be 1.5 mM, maintaining at least 90% cell viability (Supplementary Figure S3A, *https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*). In contrast, for Hs68 cells, cell viability was maintained at about 90% with 2.5 mM treatment while a decrease in cell viability to 76.4% was observed with 5 mM FA (Supplementary Figure S3B, *https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*).

3.5. Suppression of melanin production-related genes

The main pathways in melanogenesis are thought to involve G protein-coupled receptors (GPCRs) and tyrosine kinase receptors (*18*). Using HEKn and Hs68 cells, we performed RNA-seq analyses and observed little change in gene expression related to melanin production with FA treatment in Hs68 cells at all concentrations. In contrast, a significant decrease in expression of *FGF-2* gene was observed with FA treatment in HEKn cells. Since we wanted to examine the relationship between FA treatment and the tyrosine kinase signaling pathway involving FGF-2, SCF, HGF, and NRG1, we quantitatively analyzed their gene expression in HEKn cells by qRT-PCR (Figures 6A-6C). The expression of FGF-2, SCF, and NRG1 were significantly reduced after 1.0 and 1.5 mM FA treatment for 4 h, while *HGF* gene could not be detected. In the case of FGF-2, 60% inhibition was observed at 1.5 mM FA. For SCF and NRG1, 65% and 21% inhibition were observed at 1 mM FA, respectively.

3.6. Increased expression of genes that remove glycation

The accumulation of AGEs is thought to be due to glycative stress. It also affects the skin and contributes to a dull, yellowish appearance that is mediated by multiple AGEs receptors, such as RAGE (receptor for advanced glycation end products), AGE-R1, AGE-R2, and AGE-R3. RAGE and AGE-R2 particularly cause inflammation (*19*). In contrast, AGE-R1 and AGE-R3 are thought to be involved in the degradation and removal of AGEs, as also are CD36 and SR-A (scavenger receptor class A) (*20*). We performed RNA-seq analysis using HEKn cells treated with FA, examined the genes related to glycation, and observed only a change in CD36 gene expression. No significant changes in the expression of other genes related to glycation were observed. Therefore, we quantitatively analyzed the gene expressions of CD36 in HEKn cells by qRT-PCR and found an approximately three-fold increase in *CD36* gene expression with 1.5 mM FA treatment (Figure 6D).

## 4. Discussion

Several human clinical trials of FA have been reported previously. When FA, vitamin C, and vitamin E were combined, an increase in solution stability, reduction of UV-induced skin damage, and the photoprotective effects of FA were observed (*21*). Similarly, in a human clinical trial of FA alone on patients with photoaging symptoms, there was a significant improvement observed in melanin levels, erythema, and skin moisture content (*22*). To the best of our knowledge, human clinical trials examining the effect of FA on dull skin have not been reported. Therefore, we performed human clinical trials using FA by itself and exhibited the effectiveness of FA on dull skin in this study. In addition, the mechanism of FA on

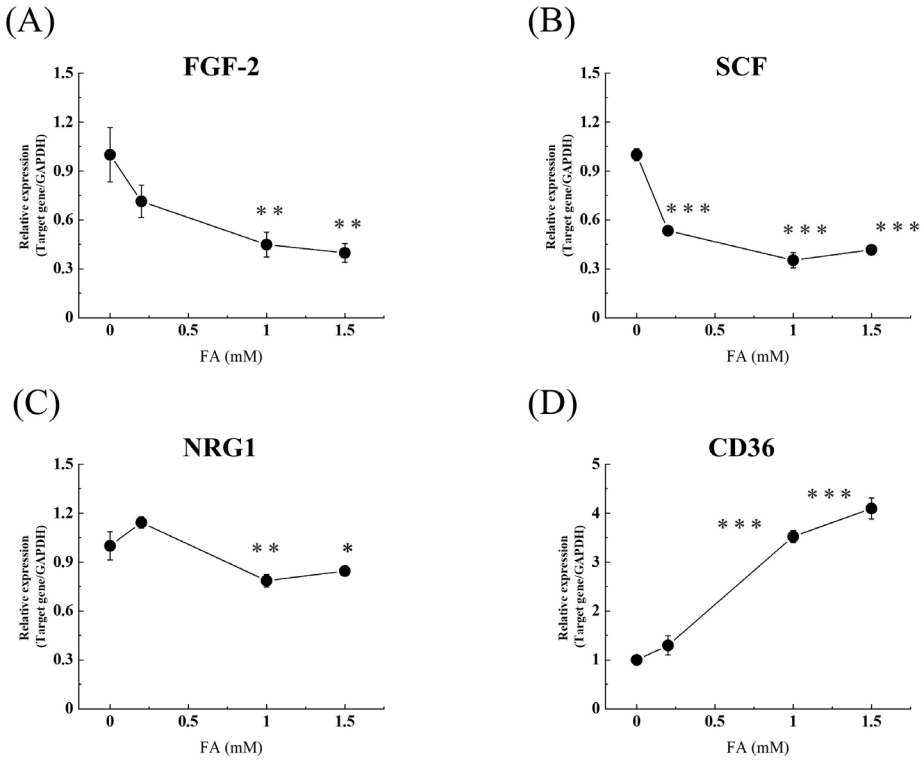


**Figure 6. Results of the gene expression analysis.** In HEKn cells, FA inhibited FGF2, SCF, and NRG1 expression, while FA facilitated CD36 expression. **(A)** Evaluation of FGF2 gene expression levels. **(B)** Evaluation of SCF gene expression levels. **(C)** Evaluation of NRG1 gene expression levels. **(D)** Evaluation of CD36 gene expression levels. The horizontal axis is the gene expression level and the vertical axis is the concentration of FA treatment. The expression level of each gene is given as a ratio to that of GAPDH. Data are expressed as means ± SEM ($n$ = 4) and were analyzed by Dunnett's test. *$p$ < 0.05, **$p$ < 0.01 ***$p$ < 0.001 (*vs.* FA-untreated samples). (FGF-2 in 1 mM FA, $p$ = 0.002; FGF-2 in 1.5 mM FA, $p$ = 0.001; SCF in 0.2 mM FA, $p$ < 0.00001; SCF in 0.2 mM FA, $p$ < 0.00001; SCF in 1 mM FA, $p$ < 0.00001; SCF in 1.5 mM FA, $p$ < 0.00001; NRG1 in 1 mM FA, $p$ = 0.009; NRG1 in 1.5 mM FA, $p$ = 0.039; CD36 in 1 mM FA, $p$ < 0.00001; CD36 in 1.5 mM FA, $p$ < 0.00001).

skin dullness at the genetic level was investigated.

Although FA has been widely noted as a cosmetic ingredient, there are stability issues due to high sensitivity to light and oxygen (*23*). These issues limit the range of FA applications in beauty and skin care products. In this study, stability tests were conducted for FA (Supplementary Figure S2, *https://www.ddtjournal.com/action/getSupplementalData.php?ID=307*) and the FA concentration for the clinical study was set at 1%. In Figure 3, a decrease in melanin levels was observed in both groups after week 4. One factor contributing to this might be that this examination was conducted from September to December when UV radiation was greatly reduced. Previous studies have reported that reduced UV exposure leads to a natural reduction in melanin production (*24*). Even in such an environment, our results suggest that the amount of melanin, one of the main causes of dull skin, was reduced in the group of subjects who continuously applied FA cream for 8 weeks, compared to week 0. However, a comparison between groups showed no statistically significant differences. This is presumably due in part to the relatively low concentration of 1% used in this study. On the other hand, from week 8 to 12, a phenomenon was observed in which the melanin indexes of the FA and placebo groups crossed. One reason for this might be winter-specific environmental factors such as low temperature and moisture in addition to the decrease in UV exposure. It has been reported that the skin barrier function tends to be impaired in winter environments such as low humidity and cold temperatures, and that external stimuli such as dryness and friction can cause inflammation (*25*). Such inflammation may cause post-inflammatory hyperpigmentation (PIH), which may slightly alter melanin levels (*26*).

A melanin suppression effect of FA was supported not only by objective indicators but also by subjective evaluation (VAS questionnaire). In the subjective evaluation, the improvement effect was confirmed in the subjects' perception of their own skin for all the other evaluation items, except for the brownish dark category. Specifically, improvement was recorded in the evaluation items of moisture, dark dullness resulting from skin dryness and stratum corneum thickening, yellowish dullness due to glycation, as well as skin dullness and translucent skin. Significant improvements were observed at week 12 in the FA group compared to week 0 (Figure 4B-4F). The results of the assessment of subjects were consistent with the changes in facial images in two representative subjects shown in Figure 5A and 5B. After the application of FA cream, the evaluation and VISIA Evolution of both subjects showed a significant

improvement in the overall dullness of the skin and an improvement in the brightness of the skin tone. These results suggested that FA might be effective in improving skin tone by acting in a complex manner against various causes of dark spots.

Melanogenesis, the cause of brownish dullness, is thought to be regulated by multiple signaling pathways, involving primarily MITF (microphthalmia-associated transcription factor) which regulates the expression and activity of melanogenic enzymes. Main signaling routes include GPCRs (*e.g.* melanocortin receptor, endothelin receptor B and frizzled receptor), tyrosine kinase receptors (*e.g.* SCF/KIT, FGF-2, and HGF signaling pathways) (*18*). To analyze the efficacy of FA at the molecular level, we performed RNA-seq using HEKn and Hs68 cells. The results indicated fluctuations in gene expression of FGF-2. We further investigated the effects of FA on the melanogenic pathway involving tyrosine kinase signaling such as SCF, FGF-2, HGF, and NRG1 using qRT-PCR. Although the *HGF* gene was not detected, decreases in gene expression of the *SCF*, *FGF2*, and *NRG1* genes were observed. FA may inhibit the melanogenic pathway *via* tyrosine kinase-type receptors (Figure 6A-6C). Furthermore, FGF-2 has been shown to activate the MAPK/ERK and PI3K/Akt pathways *via* the FGF receptor and promote keratinocyte proliferation. This proliferation might thicken the epidermis and stratum corneum thereby contributing to the dark appearance of the skin by increasing diffuse light reflection (*27,28*). Decrease in *FGF2* gene expression by FA may be involved in the suppression of stratum corneum thickening through regulation of keratinocyte growth and survival. These changes at the molecular level were also consistent with the improvement in darkening caused by stratum corneum thickening observed in human clinical trials.

AGEs were thought to cause dull, yellowish skin, and other skin problems (*8*). As shown in Figure 6D, FA treatment significantly enhanced gene expression of CD36, a receptor that degrades and removes AGEs. This result was consistent with the previous studies, indicating that FA may remove AGEs produced in glycation reactions and prevent the development of yellowish dullness (*29*).

In summary, FA demonstrates multifunctional potential in both the prevention and amelioration of different forms of dull skin, brownish dullness, yellowish dullness, and dark dullness. Thus, it may be that FA would be extremely useful as a comprehensive skincare material for improving dull skin and enhancing skin clarity. Future studies on the practical use of FA will be needed to improve the stability of the formulation for higher concentrations of FA, combined with other whitening ingredients and anti-glycation/anti-aging ingredients. Further studies should be done in different season (spring, summer). The applicability to different skin types and age groups should also be investigated.

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**References**

1. Kumar M, Kaushik D, Shubham S, Kumar A, Kumar V, Oz E, Brennan C, Zeng M, Proestos C, Çadırcı K, Bayrak M, Elobeid T, Karav S, Oz F. Ferulic acid: extraction, estimation, bioactivity and applications for human health and food. J Sci Food Agric. 2025; 105:4168-4177.
2. Taniguchi H, Nomura E, Tsuno T, Minami S, Kato K, Hayashi C. Method of manufacturing ferulic acid. U.S. Patent US5288902A. 1994 Feb 22.
3. Truong HT, Van MD, Huynh LD, Nguyen LT, Tuan AD, Thanh TLX, Phuoc HD, Takenaka N, Imamura K, Maeda Y. A method for ferulic acid production from rice bran oil soapstock using a homogenous system. Appl Sci. 2017; 7:796.
4. Zhou L, Huang J, Du Y, Li F, Xu W, Zhou C, Liu S. Non-thermal stabilization strategies for rice bran: mechanistic insights, technological advances, and implications for industrial applications. Foods. 2025; 14:1448.
5. Nakagawa S, Kobayashi M, Nakamura N, Tsuno T. Effectiveness of rice bran ferulic acid as a cosmetic ingredient. Pharmacometrics. 2022; 102:41-45.
6. Kaneko O, Tsukada H, Ishikawa Y, Kawaguchi Y. Measuring apparent darkening of the skin (first report): factors inherent in light reflected from the skin. J Soc Cosmet Chem Jpn. 1997; 31:44-51.
7. Kaneko O, Kawaguchi Y, Ishikawa Y, Inagaki K. Measuring apparent darkening of the skin (second report): relationship between age-associated changes in physical properties of the skin and apparent darkening. J Soc Cosmet Chem Jpn. 1997; 31:429-438.
8. Zheng W, Li H, Go Y, Chan XH, Huang Q, Wu J. Research advances on the damage mechanism of skin glycation and related inhibitors. Nutrients. 2022; 14:4588.
9. Correia G, Magina S. Efficacy of topical vitamin C in melasma and photoaging: a systematic review. J Cosmet Dermatol. 2023; 22:1938-1945.
10. Zduńska-Pęciak K, Kołodziejczak A, Rotsztejn H. Two superior antioxidants: ferulic acid and ascorbic acid in reducing signs of photoaging-a split-face comparative

study. Dermatol Ther. 2022; 35:e15254.
11. Jaros-Sajda A, Budzisz E, Erkiert-Polguj A. Ascorbic acid treatments as effective and safe anti-aging therapies for sensitive skin. Antioxidants. 2024; 13:174.
12. Adachi H, Murakami Y, Hotta N, Tanaka H, Nakata S. Investigation of the skin aging mechanism focusing on stratifin. J Soc Cosmet Chem Jpn. 2015; 49:211-217.
13. Seif El Nasr H, Shaker OG, Fawzi MMT, El-Hanafi G. Basic fibroblast growth factor and tumour necrosis factor alpha in vitiligo and other hypopigmented disorders: suggestive possible therapeutic targets. J Eur Acad Dermatol Venereol. 2013; 27:103-108.
14. Mildner M, Mlitz V, Gruber F, Wojta J, Tschachler E. Hepatocyte growth factor establishes autocrine and paracrine feedback loops for the protection of skin cells after UV irradiation. J Invest Dermatol. 2007; 127:2637-2644.
15. Belleudi F, Cardinali G, Kovacs D, Picardo M, Torrisi MR. KGF promotes paracrine activation of the SCF/c-KIT axis from human keratinocytes to melanoma cells. Transl Oncol. 2010; 3:80-90.
16. Kim JS, Choi IG, Lee BC, Park JB, Kim JH, Jeong JH, Jeong JH, Seo CH. Neuregulin induces CTGF expression in hypertrophic scarring fibroblasts. Mol Cell Biochem. 2012; 365:181-189.
17. Fujinami K, Dan K, Tanaka-Kagawa T, Kawamura I. Anti-aging effects of polyoxometalates on skin. Appl Sci. 2021; 11:11948.
18. Serre C, Busuttil V, Botto JM. Intrinsic and extrinsic regulation of human skin melanogenesis and pigmentation. Int J Cosmet Sci. 2018; 40:328-347.
19. Fang B, Li L, Winget J, Laughlin T, Hakozaki T. Identification of yellow advanced glycation end products in human skin. Int J Mol Sci. 2024; 25:5596.
20. Yagi M, Yonei Y. Glycative stress and anti-aging 5. Glycative stress and receptors for AGEs as ligands. Glycative Stress Res. 2017; 4:212-216.
21. Lin FH, Lin JY, Gupta RD, Tournas JA, Burch JA, Selim MA, Monteiro-Riviere NA, Grichnik JM, Zielinski J, Pinnell SR. Ferulic acid stabilizes a solution of vitamins C and E and doubles its photoprotection of skin. J Invest Dermatol. 2005; 125:826-832.
22. Zdunska-Pęciak K, Dębowska R, Kołodziejczak A, Rotsztejn H. Ferulic acid-a novel topical agent in reducing signs of photoaging. Dermatol Ther. 2022; 35:e15543.
23. Das S, Wong ABH. Stabilization of ferulic acid in topical gel formulation *via* nanoencapsulation and pH optimization. Sci Rep. 2020; 10:12288.
24. Hexsel D, Caspary P, Dini TDF, Schilling-Souza J, Siega C. Variation of melanin levels in the skin in areas exposed and not exposed to the sun following winter and summer. Surg Cosmet Dermatol. 2013; 5:298-301.
25. Engebretsen KA, Johansen JD, Kezic S, Linneberg A, Thyssen JP. The effect of environmental humidity and temperature on skin barrier function and dermatitis. J Eur Acad Dermatol Venereol. 2016; 30:223-249.
26. Davis EC, Callender VD. Postinflammatory hyperpigmentation: a review of the epidemiology, clinical features, and treatment options in skin of color. J Clin Aesthet Dermatol. 2010; 3:20-31.
27. Shipley GD, Keeble WW, Hendrickson JE, Coffey RJ Jr, Pittelkow MR. Growth of normal human keratinocytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. J Cell Physiol. 1989; 138:511-518.
28. Tsuboi M. Development of functional active compounds from natural resources. Oleo Sci. 2011; 11:155-160.
29. Yagi M, Sakiyama C, Kitaba T, Kondo H, Mamun-Or-Rashid ANM, Lucy TT, Yonei Y. Antiglycative effect of ferulic acid. Glycative Stress Res. 2022; 9:186-193.

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***Original Article***

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# Lactoferrin improves scopolamine-induced memory impairment in mice

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**SUMMARY**: Population aging is increasing worldwide, accompanied by a rising prevalence of cognitive decline and dementia, imposing substantial healthcare and socioeconomic burdens. This study evaluated the effects of lactoferrin on scopolamine-induced memory impairment in 5-week-old ddY mice using the Barnes maze test and the novel object recognition assay to assess cognitive function. Additionally, gene expression levels in the hippocampus were analyzed using reverse transcription-quantitative PCR to explore the underlying mechanisms. Scopolamine impaired spatial and object recognition memory, whereas lactoferrin markedly improved these deficits, with effects comparable to those of donepezil. Scopolamine also increased inflammatory and oxidative stress markers (*Tnf and Nos2*) and the apoptosis-related factor (*Casp3*), all of which were attenuated by lactoferrin. These findings indicate that lactoferrin improved scopolamine-induced memory impairment, potentially by suppressing inflammation and oxidative stress, thereby inhibiting neuronal apoptosis. Therefore, lactoferrin may represent a promising functional food component for the prevention of cognitive decline.

***Keywords***: Lactoferrin, scopolamine, memory, *Nos2*, *Casp3*

## 1. Introduction

Population aging is a global issue that not only leads to a decline in the labor force but also increases the risk of various diseases. With advanced age, the likelihood of becoming bedridden increases owing to fractures and muscle weakness. Among central nervous system disorders, dementia is a major concern and is often accompanied by behavioral and psychological symptoms, such as wandering and delusions, which contribute to increasing healthcare costs.

Diseases associated with memory impairment include Alzheimer's disease and Lewy body dementia. Although their etiologies differ, one leading hypothesis is the loss of cholinergic neurons projecting to the limbic system, including the hippocampus. This neuronal loss reduces the amount of acetylcholine released into the synaptic clefts. Consequently, drugs inhibiting cholinesterase, the enzyme responsible for breaking down acetylcholine, are widely used to treat dementia. Nerve cells typically degenerate with age; however, a decrease in acetylcholine has been reported to increase oxidative stress in neurons (*1*), thereby accelerating neuronal degeneration.

Mouse models are essential for evaluating the efficacy of compounds against memory impairment. While SAMP8 mice exhibit age-related decline, scopolamine-induced models enable rapid and reproducible impairment through cholinergic blockade, making them valuable for pharmacological studies. In addition, numerous studies have used memory impairment models induced by scopolamine, a muscarinic receptor antagonist (*2-4*). In a previous study, we demonstrated that scopolamine administration caused memory impairment in the Barnes maze test and reported the involvement of oxidative stress associated with increased *Nos2* levels (*5*). Considering the increasing medical costs associated with the growing number of patients, it is important to focus not only on the pharmacological treatment of dementia but also on its prevention through the daily intake of functional foods and bioactive ingredients.

Lactoferrin (LF) has multiple biological functions, including anti-inflammatory, antibacterial, and antiviral activities, as well as beneficial effects on lipid metabolism (*6-8*). LF has been widely utilized as a functional food ingredient in Japan (*9*) and is included

as a functional component under the Food with Function Claims system regulated by the Consumer Affairs Agency. In addition to its role in immune regulation (*10*), LF has attracted attention owing to its potential effects on the central nervous system. Previous studies have shown that LF enhances analgesic responses and exhibits antidepressant-like effects (*11,12*). Furthermore, our group has demonstrated that it ameliorates suppression of the serotonergic system in ovariectomized mouse models (*13*). At the cellular level, LF has been shown to promote neuronal cell growth by activating extracellular signal-regulated kinase signaling in PC12 cells (*14*). Moreover, accumulating evidence suggests that LF exerts protective effects against tissue damage by suppressing inflammation and oxidative stress, as demonstrated in a model of nonalcoholic steatohepatitis (NASH) (*15*). Given that oxidative stress is a key contributor to memory impairment, including scopolamine-induced memory impairment, these findings suggest that LF may exert protective effects against cognitive dysfunction.

Therefore, we hypothesized that LF would attenuate scopolamine-induced cognitive impairment through neuroprotective mechanisms that suppress oxidative stress, neuroinflammation, and neuronal cell death–related signaling pathways. To test this hypothesis, we investigated the effects of LF on cognitive function and markers associated with neuroprotective pathways in a scopolamine-induced mouse model.

## 2. Materials and Methods

### 2.1. Animals

We employed 5-week-old ddY male mice purchased from SLC Inc. (Shizuoka, Japan). The animals were housed under controlled conditions (24 ± 1°C and 55% humidity) and maintained on a 12-h light/12-h dark cycle (light period, 07:00-19:00, dark period, 19:00-07:00). The animals were fed a standard rodent diet (Labo MR Stock; Nosan, Kanagawa, Japan) and provided tap water *ad libitum*. All experiments were conducted in accordance with the Guidelines for the Proper Conduct of Animal Experiments (Science Council of Japan) and were approved by the Yokohama University of Pharmacy (2023-014).

The mice were allocated to four experimental groups: control ($n$ = 24), scopolamine hydrobromide trihydrate (SCOP; $n$ = 24), SCOP + LF ($n$ = 24), and SCOP + donepezil hydrochloride (DNP; $n$ = 14). Behavioral experiments were conducted in three independent experimental batches owing to limitations in the number of animals that could be assessed simultaneously. The control, SCOP, and SCOP + LF groups were included in all three batches, whereas the SCOP + DNP group was included only in the second and third batches. As a result, the number of animals in the SCOP + DNP group was lower than that in the other groups. RT-qPCR analysis was performed using hippocampal tissues obtained from animals in the second and third experimental batches. This strategy enabled all samples to be analyzed under identical experimental conditions and minimized inter-assay variability.

After a 7-day acclimation period, behavioral training was initiated when the mice were approximately 6 weeks old. Following the 3-day training period, mice received the assigned treatments for 21 days. Behavioral testing and tissue collection were subsequently performed at 9–10 weeks of age (Figure 1).

### 2.2. Administration of experimental compounds

SCOP and DNP were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Bovine LF was obtained from NRL Pharma, Inc. (Tokyo, Japan). SCOP was diluted in physiological saline (0.9% w/v NaCl), and LF and DNP were diluted in tap water. Animals were administered SCOP (5 mg/kg) *via* intraperitoneal injection, and LF (300 mg/kg) and DNP (3 mg/kg) were administered orally. Administration was performed for 21 consecutive days, beginning the day after completion of the Barnes maze training period. On the day of behavioral testing or necropsy, experimental compounds were administered 1 h before the respective procedure. The doses were selected based on our previous experimental findings and published evidence (*5*).

### 2.3. Barnes maze test

The Barnes maze test was performed as described in our previous study (*5*). The Barnes maze test was conducted using an MB-10 apparatus (SHINFACTORY, Fukuoka, Japan). The apparatus consisted of a circular platform measuring 455 mm in diameter and 920 mm in height, with 20 holes (25 mm diameter) located 30 mm from the edge. The escape box measured 130 mm × 95 mm × 50 mm (width × depth × height). The light intensity was maintained at 600–700 Lx as a weakly aversive stimulus. To prevent interference with learning, the maze was enclosed by partitions, and three visual cues (stars, triangles, and double-circle shapes) were attached to the partitions. To prevent the use of olfactory cues and maze orientation for task performance, the platform was rotated 90° after each trial. The maze and associated apparatus were cleaned with 70% ethanol after each trial. All procedures were recorded using a high-definition digital camera (HC-W580M; Panasonic, Tokyo, Japan). The Barnes maze test consisted of three phases: habituation, repeated learning training, and probe testing.

#### 2.3.1. Habituation

The habituation phase was conducted one day prior to the repeated learning test. During habituation, the

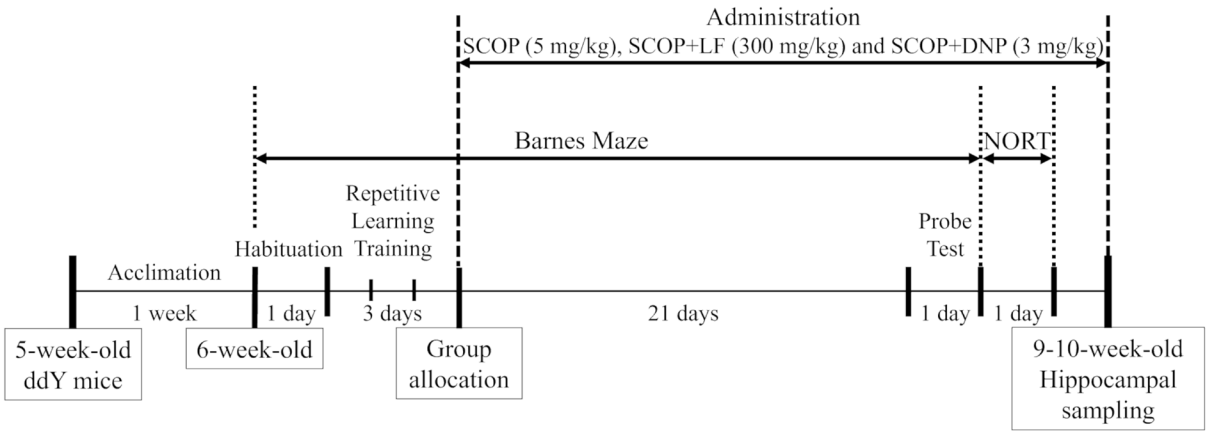


**Figure 1. Experimental timeline of the study.** Timeline of the experimental procedures, including behavioral assessments, experimental compound administration, and tissue collection. Following a 1-week acclimation period, 6-week-old male ddY mice were evaluated using the Barnes Maze test. The Barnes maze protocol consisted of a 1-day habituation phase followed by 3 days of repeated learning training. Mice were assigned to experimental groups based on their performance during the training period. Experimental compound administration began the day after completion of the Barnes maze training period and continued for 21 days. A probe test was conducted after the treatment period to assess spatial memory retention, followed by the NORT the following day. Hippocampal tissues were subsequently collected for molecular analyses. NORT, novel object recognition test; SCOP, scopolamine hydrobromide trihydrate (5 mg/kg); SCOP + LF, SCOP plus lactoferrin (300 mg/kg); SCOP + DNP, SCOP plus donepezil hydrochloride (3 mg/kg).

maze board was used without visual cues or an escape box, allowing the mice to explore freely for 120 s. Subsequently, each mouse was placed in the center of the maze and confined within a transparent beaker for 20 s, after which the escape box was introduced. The mouse was then guided into the escape box, with the hole blocked with an opaque weight, and remained there for 60 s. This procedure was performed once for each mouse.

2.3.2. Repetitive learning training

Repeated learning training was conducted for three consecutive days beginning the day after the habituation phase. During repeated learning training, the visual cues were displayed, and the escape box was positioned beneath the target hole. Mice were confined for 20 s in a start box (a light-proof white cylinder; 9.7 cm in diameter and 13.2 cm in height) positioned at the center of the maze. The start box was then removed, and each mouse was allowed to freely explore the maze for up to 120 s. During this time, the latencies to locate and enter the escape box were recorded independently. If a mouse did not enter the escape box within the allotted time, it was guided to the escape box. Upon entering the escape box, the mouse was confined for 60 s. After completion of the training period, the learning rate was calculated by dividing the mean escape box arrival latency on day 3 by that on day 1. Prior to treatment allocation, animals were assigned to four groups (control, SCOP, SCOP + LF, and SCOP + DNP), and no significant differences in learning rate were observed among the groups.

2.3.3. Probe test

The probe test was conducted 21 days after completion of repeated learning training. During the probe test, visual cues were present, whereas the escape box was removed. The mice were confined in the start box at the center of the maze for 20 s, after which they were allowed to explore the maze freely for 120 s. Behavior was recorded using a camera positioned directly above the maze. This procedure was performed once for each mouse. Only the first 60 s of the recorded behavior were included in the analysis. The maze board was divided into four zones: the area around the escape box and associated star-shaped visual cue was designated ZONE1; the area surrounding the triangle-shaped visual cue was designated ZONE2; the area surrounding the double-circle visual cue was designated ZONE3; and the area without visual cues was designated ZONE4. The time spent in each zone and the proportion of total distance traveled within each zone were measured to evaluate spatial memory.

2.4. Novel object recognition test (NORT)

The NORT was performed as previously described (*16,17*). This experiment was conducted in an open-field apparatus (50 × 50 × 30 cm high) under a light intensity of 25–50 lx. Two objects of similar height were selected: object A (8.2 cm in diameter, 9.7 cm in height; white cylinder) and object B (6.5 cm in diameter, 8.9 cm in height; gray cylinder), which differed in color and shape. The object was placed along the diagonal axis within the central 25 × 25 cm area of the open field. To minimize the influence of olfactory or positional cues, object positions were swapped for each mouse. The maze and objects were cleaned with 70% ethanol between trials. All procedures were recorded using a high-definition

digital camera. The NORT consisted of three phases: habituation, familiarization (T1), and testing (T2), with experimental compounds administered 1 h before each phase.

During habituation, mice were allowed to freely explore the open field for 10 min in the absence of objects. Familiarization (T1) was conducted the day after habituation. During T1, two identical objects were placed, and each mouse was released into the center of the arena and allowed to explore freely for 10 min. T2 was conducted 1 h after T1. During T2, one of the identical objects used in T1 was placed at the same location as during T1, whereas the other object was replaced with a novel object and positioned at the corresponding location. Mice were released into the center of the open field and allowed to explore freely for 10 min. Only the first 5 min of the exploration period were included in the analysis. The duration and frequency of object exploration were recorded for each object. For the novel object, the exploration duration and exploration frequency were each divided by the corresponding total value for the familiar and novel objects combined, and the resulting values were expressed as percentages. Two animals in the SCOP + DNP group died during the oral administration period owing to an inadvertent gavage-related technical error and were excluded from the NORT analysis.

2.5. Reverse transcription-quantitative PCR (RT-qPCR)

The hippocampus was dissected from mouse brains, and total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan). Tissue homogenization was performed using a Polytron PT 1300 D homogenizer (Kinematica AG, Lucerne, Switzerland). Complementary DNA (cDNA) was synthesized using PrimeScript™ RT Master Mix (Takara Bio Inc., Shiga, Japan) with the extracted RNA as a template. The reaction mixture was incubated at 37°C for 15 min, followed by incubation at 85°C for 5 s.

**Table 1. Primer sequences**

| Gene | Sequences (5'→3') |
|---|---|
| *Gapdh* | Forward: AGCTTGTCATCAACGGGAAG |
| | Reverse: TTTGATGTTAGTGGGGTCTCG |
| *Tnf* | Forward: CCTCTTCTCATTCCTGCTTGTG |
| | Reverse: GCCATTTGGGAACTTCTCATCC |
| *Il1b* | Forward: AGTTGACGGACCCCAAAAG |
| | Reverse: AGCTGGATGCTCTCATCAGG |
| *Ccl2* | Forward: GGGACACTGGCTGCTTGT |
| | Reverse: GTTGTTAAGCAGAAGATTCACGTC |
| *Nos2* | Forward: CTTTGCCACGGACGAGAC |
| | Reverse: TCATTGTACTCTGAGGGCTGAC |
| *Trp53* | Forward: CCAGGATGTTGCAGAGTTGTT |
| | Reverse: GCAGGAGCTGACACTTGGA |
| *Casp3* | Forward: GAGGCTGACTTCCTGTATGCTT |
| | Reverse: AACCACGACCCGTCCTTT |
| *Bdnf* | Forward: AGTCTCCAGGACAGCAAAGC |
| | Reverse: TGCAACCGAAGTATGAAATAACC |

RT-qPCR was performed using the LightCycler® 96 System (Hoffmann-La Roche, Basel, Switzerland) with SYBR GREEN I MASTER Mix (Hoffmann-La Roche, Basel, Switzerland), and amplification was monitored using the intercalation method. The qPCR cycling conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 40–50 amplification cycles of 95°C for 10 s (denaturation) and 60°C for 20–30 s (annealing/extension).

Gene expression levels were calculated relative to those of *Gapdh*. The PCR primer sequences are listed in Table 1.

2.6. Statistical analysis

For group comparisons, data from repeated learning training were analyzed using a linear mixed-effects model (LMM), whereas other data were analyzed using Dunnett's test. Statistical analysis was performed using the free statistical software EZR (v.1.68; Jichi Medical University, Tochigi, Japan) (*18*). Significance levels were set at $p < 0.001$, $p < 0.05$, and $p < 0.01$.

## 3. Results

3.1. Barnes maze test

The Barnes maze test was used to evaluate hippocampus-dependent spatial learning and long-term memory. Figure 2 shows the latency to enter the target hole during repetitive learning training (Figure 2). LMM analysis revealed no significant differences among the experimental groups, indicating comparable baseline performance prior to treatment allocation.

Three weeks after the initiation of experimental compound administration, a probe test was conducted.

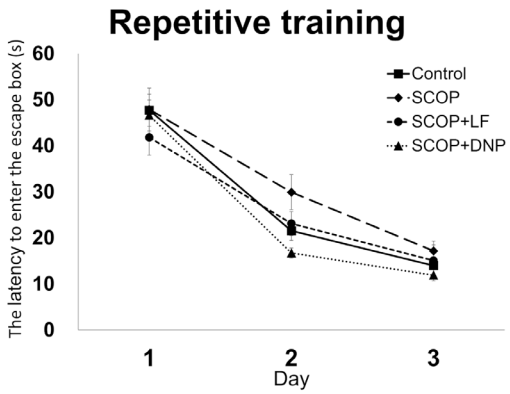


**Figure 2. Baseline learning performance during the 3-day repeated learning training period used for group allocation.** Linear mixed-effects model (LMM) analysis shows no significant between-group differences in learning rates during the repeated learning test. Data are presented as mean ± SEM (control, SCOP, and SCOP + LF: $n = 24$, SCOP + DNP: $n = 14$). SCOP, scopolamine hydrobromide trihydrate (5 mg/kg); SCOP + LF, SCOP plus lactoferrin (300 mg/kg); SCOP + DNP, SCOP plus donepezil hydrochloride (3 mg/kg).

The time spent in each zone and the percentage of distance traveled are presented in Figures 3A–3C. In ZONE1, where the target hole was located, the time spent was significantly decreased in the SCOP group (13.4 ± 1.3%) compared with the control group (23.9 ± 1.5%), whereas it was significantly increased in the SCOP + DNP group (22.0 ± 2.9%) compared with the SCOP group (Figure 3B). These findings support the successful establishment of the scopolamine-induced memory impairment model. The SCOP + LF group (22.5 ± 2.1%) also exhibited a significant increase compared with the SCOP group. In ZONE3, which is located diagonally opposite ZONE1, the control and SCOP + LF groups spent significantly less time than the SCOP group.

In ZONE1, the percentage of distance traveled was significantly reduced in the SCOP group (25.0 ± 2.3%) compared with the control group (36.7 ± 2.1%), whereas it was significantly increased in the SCOP + LF group (35.6 ± 3.0%) compared with the SCOP group (Figure 3C). Furthermore, consistent with the findings for time spent in each zone (Figure 3B), both the control and SCOP + LF groups showed a significantly lower percentage of distance traveled in ZONE3 than the SCOP group.

### 3.2. NORT

The NORT was conducted to evaluate short-term object recognition memory (Figure 4A). During the familiarization phase (T1), in which two identical objects were presented, both the object exploration duration (%) (Figure 4B) and object exploration frequency (%) (Figure 4C) were approximately 50% across all groups, indicating no object preference (Figure 4B and 4C). These results confirmed that the experimental conditions for NORT were appropriately established.

During the test phase (T2), conducted 1 h after T1, one of the familiar objects was replaced with a novel object. The novel object exploration duration (%) was significantly lower in the SCOP group than in the control group and significantly higher in the SCOP + DNP group than in the SCOP group (Figure 4B). In addition, the SCOP + LF group exhibited a significant increase in the novel object exploration duration (%) compared with the SCOP group (Figure 4B). In contrast, novel object

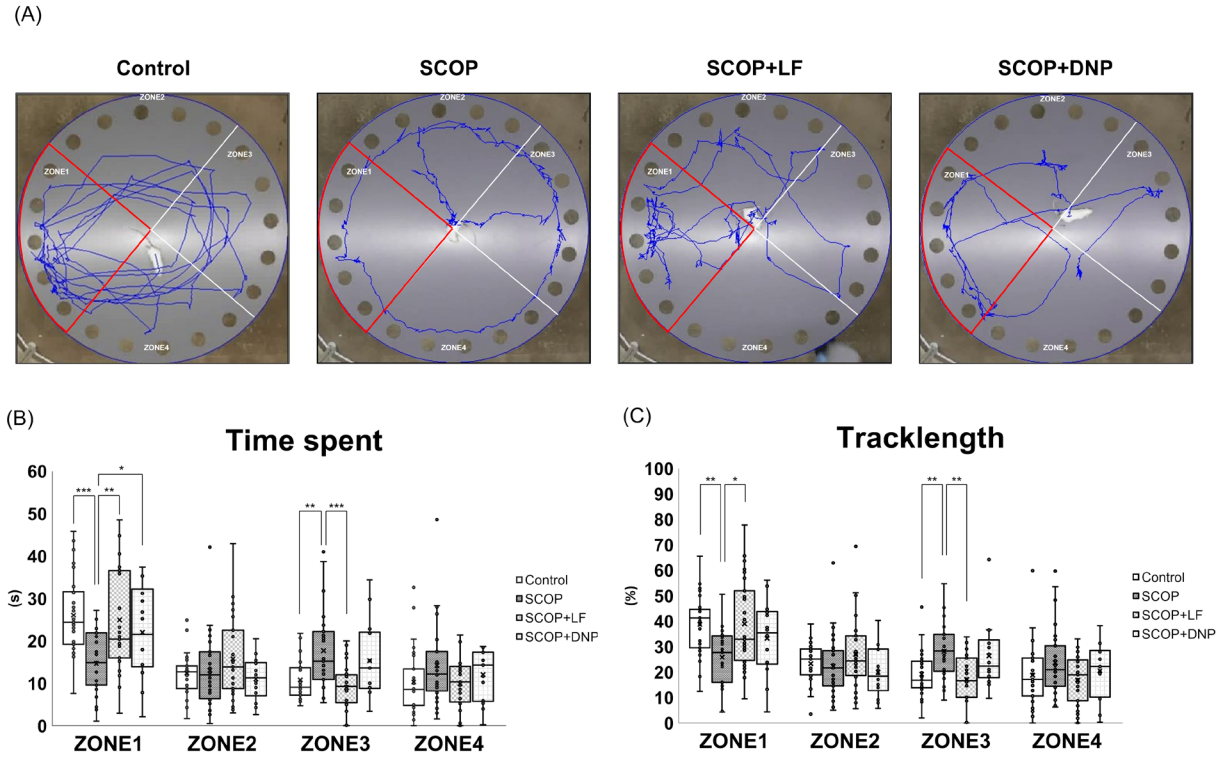


**Figure 3. Effect of 3-week experimental compound administration on Barnes maze performance.** (**A**) Representative movement trajectories during the probe test for each group (control, SCOP, SCOP + LF, and SCOP + DNP), illustrating differences in spatial exploration patterns. ZONE1 (red frame) corresponds to the area surrounding the former location of the escape box. (**B**) Time spent and (**C**) percentage of distance traveled in each zone during the Barnes maze probe test across experimental groups. Data are presented as box-and-whisker plots. The center line indicates the median, the box represents the interquartile range (25th–75th percentiles), and the whiskers indicate the minimum and maximum values (control, SCOP, and SCOP + LF: $n = 24$, SCOP + DNP: $n = 14$). Statistical analysis was performed using Dunnett's test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus SCOP. SCOP, scopolamine hydrobromide trihydrate (5 mg/kg); SCOP + LF: SCOP plus lactoferrin (300 mg/kg); SCOP + DNP, SCOP plus donepezil hydrochloride (3 mg/kg).

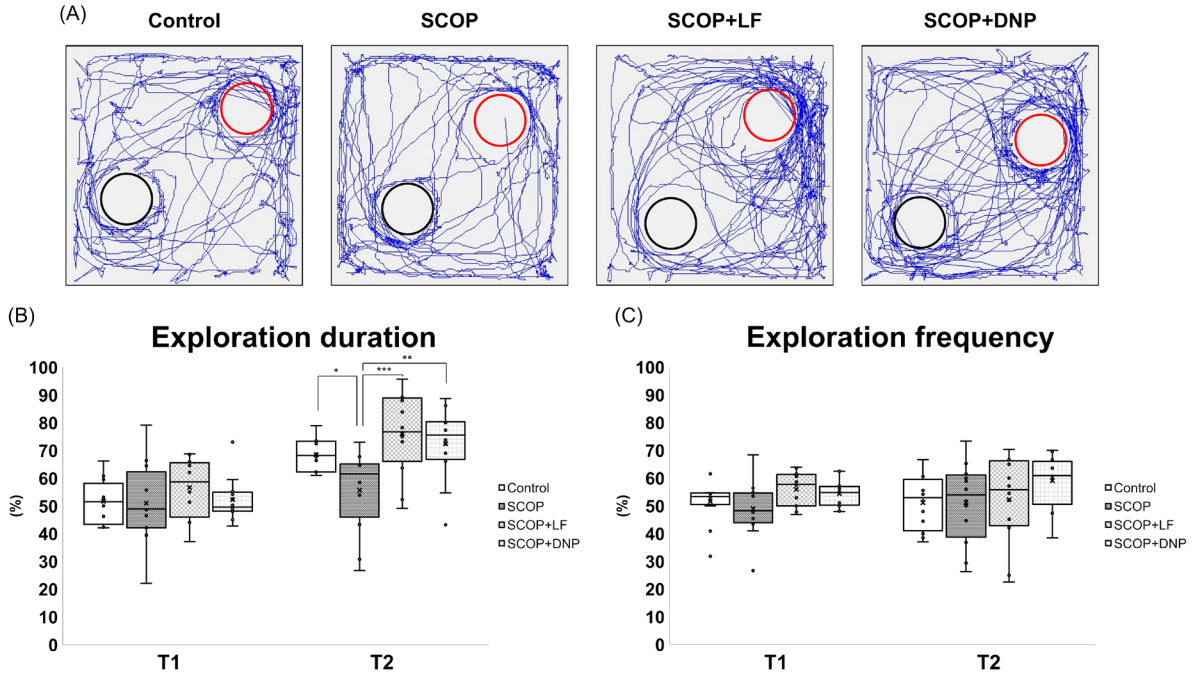


**Figure 4. Effects of experimental compound administration on short-term object recognition memory assessed 1 h after administration.** (**A**) Representative movement trajectories during the T2 in each group (control, SCOP, SCOP + LF, and SCOP + DNP), illustrating differences in spatial exploration behavior. Red circles indicate the novel object, while black circles indicate the familiar object used in T1. (**B**) Novel object exploration (%) and (**C**) novel object exploration frequency (%) in T1 and T2 of the novel object recognition test. Data are presented as box-and-whisker plots. The center line indicates the median, the box represents the interquartile range (25th–75th percentiles), and the whiskers indicate the minimum and maximum values (control, SCOP, and SCOP + LF: $n = 24$, SCOP + DNP: $n = 14$). Statistical analysis was performed using Dunnett's test. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus SCOP. SCOP, scopolamine hydrobromide trihydrate (5 mg/kg); SCOP + LF: SCOP plus lactoferrin (300 mg/kg); SCOP + DNP, SCOP plus donepezil hydrochloride (3 mg/kg).

exploration frequency (%) did not differ significantly among the groups (Figure 4C).

### 3.3. Effects of experimental compound administration on hippocampal gene expression

To evaluate the effects of the experimental compounds on hippocampal gene expression, mRNA expression levels of inflammatory mediators *Tnf*, *Il1b*, and *Ccl2*, as well as *Nos2*, a key enzyme involved in inflammation-associated nitric oxide production, were analyzed. Furthermore, *Bdnf*, a key regulator of neuronal survival and synaptic plasticity, and the apoptosis-related factors *Trp53* and *Casp3* were examined.

*Tnf* expression was significantly elevated in the SCOP group compared with the control group, and this increase was significantly attenuated in the SCOP + LF and SCOP + DNP groups (Figure 5A). In contrast, *Il1b* and *Ccl2* expression levels did not differ significantly among the groups (Figures 5B and 5C). Similarly, *Nos2* expression was significantly elevated in the SCOP group, and this increase was significantly attenuated in the SCOP + LF and SCOP + DNP groups (Figure 5D). No significant differences were observed in *Bdnf* expression levels among the groups (Figure 6A). In addition, no significant differences in *Trp53* expression levels were observed between the SCOP, control, and SCOP + DNP groups. However, *Trp53* expression was significantly reduced in the SCOP + LF group (Figure 6B). Conversely, *Casp3* expression was significantly elevated in the SCOP group, and this increase was significantly attenuated in the SCOP + LF and SCOP + DNP groups (Figure 6C).

## 4. Discussion

Our findings demonstrate that oral LF administration ameliorated scopolamine-induced memory impairment. This study was performed using a murine model of memory impairment induced by scopolamine, a muscarinic receptor antagonist. Our previous study demonstrated that scopolamine administration after repeated training induced memory impairment in the Barnes maze test (*5*). Moreover, we previously reported that SAMP8 mice, a model of accelerated aging, exhibit impaired performance during repeated learning in the Barnes maze test (*19*). Therefore, in the present study, mice were assigned to experimental groups based on their performance during the 3-day training period to ensure comparable baseline learning ability and minimize differences in memory consolidation among the groups. Following the 3-day training period, mice received scopolamine alone, scopolamine plus donepezil, or scopolamine plus LF for three weeks. In the probe test

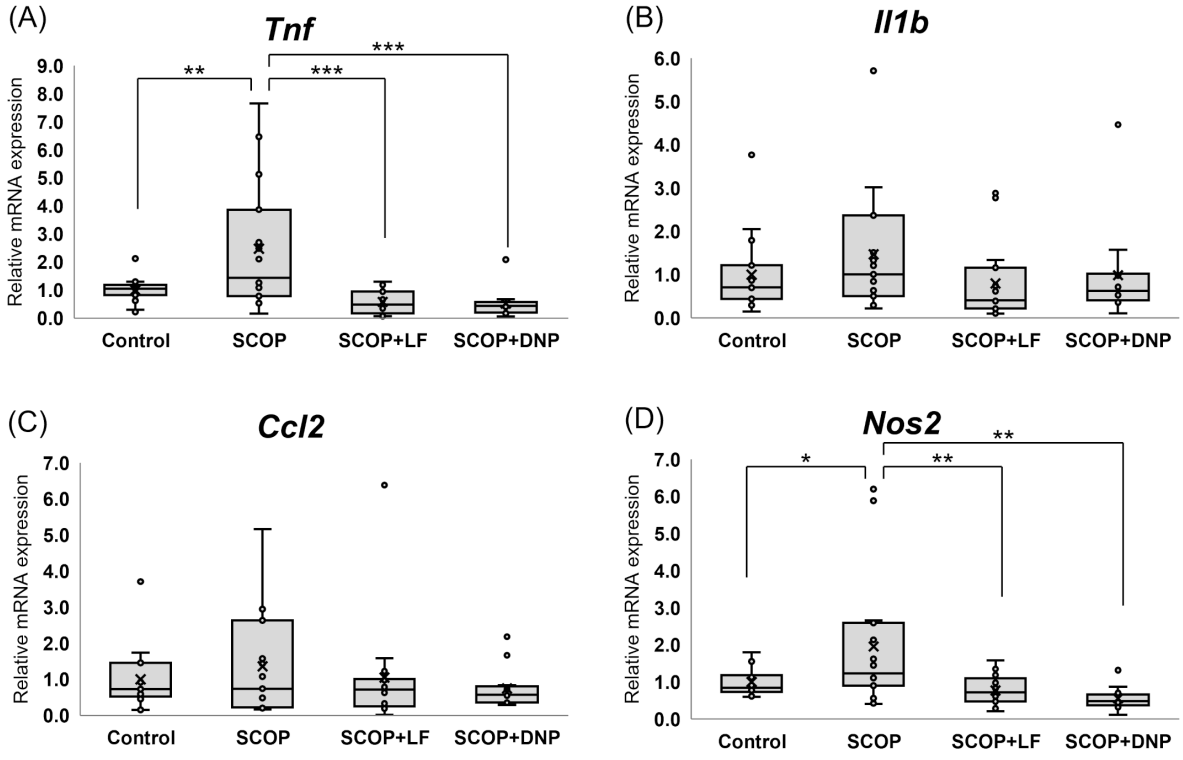


**Figure 5. Effects of experimental compound administration on hippocampal mRNA expression of Tnf, Il1b, Ccl2, and Nos2.** Hippocampal mRNA expression of inflammation- and oxidative stress-related genes. (**A**) *Tnf*, (**B**) *Il1b*, (**C**) *Ccl2*, and (**D**) *Nos2*, as quantified by RT-qPCR. Data are presented as box-and-whisker plots. The center line indicates the median, the box represents the interquartile range (25th–75th percentiles), and the whiskers indicate the minimum and maximum values (control, SCOP, and SCOP + LF: $n = 24$, SCOP + DNP: $n = 14$). Statistical analysis was performed using Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus SCOP. SCOP, scopolamine hydrobromide trihydrate (5 mg/kg); SCOP + LF: SCOP plus lactoferrin (300 mg/kg); SCOP + DNP, SCOP plus donepezil hydrochloride (3 mg/kg).

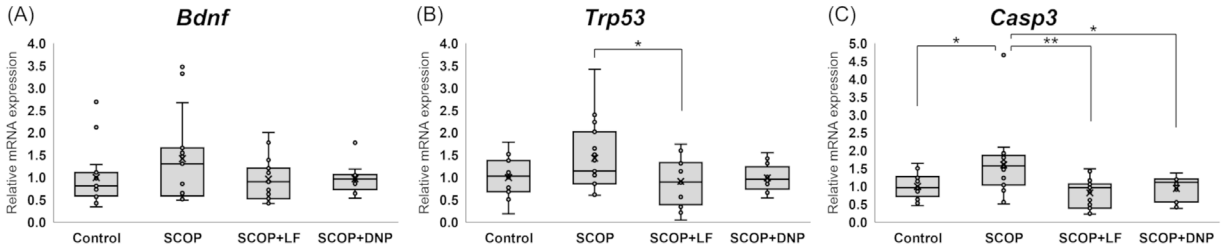


**Figure 6. Effects of experimental compound administration on hippocampal mRNA expression of *Bdnf*, *Trp53*, and *Casp3*.** Hippocampal mRNA expression levels of **(A)** *Bdnf*, a gene associated with neuronal survival and synaptic plasticity, and the apoptosis-related genes **(B)** *Trp53* and **(C)** *Casp3*, as quantified by RT-qPCR. Data are presented as box-and-whisker plots. The center line indicates the median, the box represents the interquartile range (25th–75th percentiles), and the whiskers indicate the minimum and maximum values (control, SCOP, and SCOP + LF: $n = 24$, SCOP + DNP: $n = 14$). Statistical analysis was performed using Dunnett's test. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus SCOP. SCOP, scopolamine hydrobromide trihydrate (5 mg/kg); SCOP + LF: SCOP plus lactoferrin (300 mg/kg); SCOP + DNP, SCOP plus donepezil hydrochloride (3 mg/kg).

conducted after the treatment period, the control group demonstrated sustained memory retention, spending approximately 24% of the test time in ZONE1 even two weeks after training. In contrast, the scopolamine-only group spent only 13% of the test time in ZONE1, indicating impaired memory retention.

Cholinergic neurons projecting from the Meynert basal nucleus to the hippocampus and other brain regions are involved in memory processes, and scopolamine induces memory impairment by blocking muscarinic receptors (*20*). Accordingly, we postulated that the scopolamine-induced memory impairment was mediated, at least in part, through this pathway. In this study, donepezil, a cholinesterase inhibitor widely used for the treatment of Alzheimer's disease, markedly improved memory impairment, suggesting that restoration of cholinergic neurotransmission can counteract the effects of scopolamine-induced muscarinic receptor blockade.

Although LF does not possess cholinesterase-inhibitory activity, it produced a comparable improvement in memory impairment. Analysis of trajectories in each region during the probe test revealed that activity in the region opposite to the target region was significantly reduced in the LF group. In contrast, the donepezil group exhibited a more uniform distribution of activity across the three non-target regions, suggesting that donepezil may improve memory performance through distinct underlying mechanisms.

In the NORT (T2), the scopolamine-only group demonstrated a novel object exploration duration (%) of approximately 50%, suggesting impaired short-term recognition memory. In contrast, the LF and donepezil groups exhibited novel object exploration durations of ≥ 70%, suggesting that these treatments improved short-term memory impairment. Notably, no significant differences were observed in novel object exploration frequency (%) among the groups, supporting the validity of the object recognition memory test. To investigate the mechanisms underlying the LF-mediated effects on memory, hippocampal gene expression levels were analyzed by RT-qPCR. Scopolamine administration has been shown to induce inflammation *via* muscarinic receptor blockade (*21,22*). In addition, scopolamine administration has been reported to markedly increase *Nos2* levels, which are associated with oxidative stress (*5*). LF has been reported to exert anti-inflammatory effects in immune cells (*23*), and our previous study using a NASH mouse model demonstrated its ability to suppress hepatic inflammation (*15*). In addition, LF has been reported to possess antioxidant properties (*24*). To investigate the effects of scopolamine-induced inflammation in the hippocampus, we examined the expression of *Tnf, Il1b*, and *Ccl2*. Scopolamine administration markedly increased *Tnf* expression, whereas LF and donepezil significantly suppressed this increase, suggesting anti-inflammatory effects. TNF-α is a proinflammatory cytokine produced primarily by macrophages. Liu *et al.* (2022) reported that LF exerts anti-inflammatory effects by modulating macrophage function (*25*), raising the possibility that a similar mechanism may also operate in the hippocampus. In the central nervous system, microglia perform functions analogous to those of macrophages; therefore, LF may exert anti-inflammatory effects through the modulation of microglial activity. Our previous study using a NASH mouse model demonstrated that LF suppresses inflammation through effects on hepatic macrophages, and the findings of the present study further support the anti-inflammatory properties of LF. In contrast, although *Il1b* demonstrated a trend similar to that of *Tnf*, no statistically significant difference was observed. Likewise, no significant differences were observed in the expression of *Ccl2*, a chemokine involved in macrophage migration. One possible explanation for this finding is that hippocampal tissue was collected 24 h after the final dose. Next, we examined the expression of *Nos2*, which encodes inducible nitric oxide synthase (iNOS), a key enzyme involved in inflammation-associated oxidative stress. iNOS catalyzes the production of nitric oxide, which activates soluble guanylate cyclases. In turn, reactive oxygen and nitrogen species induce *Nos2* expression, establishing a positive feedback loop. Thus, *Nos2* upregulation may contribute to the exacerbation of oxidative stress. Scopolamine markedly increased the expression of *Nos2*, whereas LF or donepezil substantially inhibited this increase. Likewise, our previous study demonstrated that scopolamine increases oxidative stress (*5*). Furthermore, scopolamine has been reported to enhance oxidative stress by inhibiting superoxide dismutase activity (*26*); however, the mechanisms underlying scopolamine-induced oxidative stress remain to be fully elucidated. Because LF suppressed the scopolamine-induced increase in oxidative stress, we next examined the expression of *Bdnf*, a key regulator of neuronal survival and synaptic plasticity, as well as the apoptosis-related genes *Trp53* and *Nos2*. Although *Bdnf* expression did not differ significantly among the groups, it tended to increase in the scopolamine group and decrease following LF treatment. Elevated BDNF levels promote nerve growth, whereas oxidative stress is generally thought to inhibit this process. However, despite the increase in oxidative stress induced by scopolamine, *Bdnf* expression tended to increase in the present study. One possible explanation is that the relatively young age of the mice (approximately 10 weeks old) elicited a feedback mechanism. Wu *et al*. (2020) reported that BDNF suppression contributed to neuronal loss in 24-week-old mice (*27*), suggesting that age may influence the relationship between oxidative stress and *Bdnf* expression. The scopolamine-treated group exhibited a trend toward increased *Trp53* expression and a significant increase in *Casp3* expression. This suggests that scopolamine induces inflammation by activating macrophages and promotes neuronal cell death by increasing oxidative stress. Because LF markedly suppressed these changes, it may reduce oxidative stress in neurons and exert anti-inflammatory effects by modulating microglial activity.

The beneficial effects of LF on cognitive function have previously been reported in aged mice and *APP/PS1* transgenic mouse models (*28,29*). Consistent with these findings, the present study demonstrated that LF improves memory performance. However, unlike previous studies, we employed a scopolamine-induced cognitive impairment model, which is widely used to mimic cholinergic dysfunction–associated memory deficits. Furthermore, the beneficial effects of orally administered LF were associated with the reduced expression of *Tnf*, *Nos2*, *Trp3*, and *Casp3*. Accordingly, LF may ameliorate cognitive impairment by modulating neuroinflammatory, oxidative stress-related, and

apoptosis-related pathways during cholinergic dysfunction.

These results indicate that LF improves scopolamine-induced memory impairment by inhibiting neuronal loss *via* its anti-inflammatory and antioxidant effects.

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**References**

1. Wal P, Dutta A, Jawaid T, *et al*. Synaptic aging and neurodegeneration: the role of synaptic vesicle dynamics and neurotransmitter imbalance. Biogerontology. 2026; 27:55.
2. Zavala-Ocampo LM, López-Camacho PY, Aguirre-Hernández E, Cárdenas-Vázquez R, Bonilla-Jaime H, Basurto-Islas G. Neuroprotective effects of Petiveria alliacea on scopolamine-induced learning and memory impairment mouse model. J Ethnopharmacol. 2024; 318:116881.
3. Kim SS, Kim WS, Moon H, Oh SJ, Hong GS, Lee B, Choi CW, Lee B, Choi JS, Kim MS. 3',4',7-trihydroxyflavone activates the CREB-BDNF axis and restores scopolamine-induced memory deficit in mice. Eur J Pharmacol. 2025; 999:177645.
4. Jee SC, Lee KM, Kim M, Lee YJ, Kim S, Park JO, Sung JS. Neuroprotective effect of *Cudrania tricuspidata* fruit extracts on scopolamine-induced learning and memory impairment. Int J Mol Sci. 2020; 21:9202.
5. Ishiyama Y, Furukawa M, Toho M, Aoki R, Fujimura R, Shibuya M, Sato K, Nagashima D, Izumo N. The Barnes maze test is useful for evaluating scopolamine-induced memory impairment model mice. Pharmacometrics. 2025; 108:169-176.
6. Rosa L, Ianiro G, Conte AL, Conte MP, Ottolenghi L, Valenti P, Cutone A. Antibacterial, anti-invasive, and anti-inflammatory activity of bovine lactoferrin extracted from milk or colostrum versus whole colostrum. Biochem Cell Biol. 2024; 102:331-341.
7. Ding L, Chen JS, Xing YF, Li DM, Fu AQ, Tong X, Chen GC, Xu JY, Qin LQ. Effects of lactoferrin on high-fat and high-cholesterol diet-induced non-alcoholic fatty liver disease in mice. J Nutr Biochem. 2025; 143:109938.
8. Rizzi M, Manzoni P, Germano C, Quevedo MF, Sainaghi PP. Lactoferrin, a natural protein with multiple functions in health and disease. Nutrients. 2025; 17:3403.
9. Ueno H. Applications of lactoferrin as a functional food ingredient. Jpn J Dairy Sci. 2012; 61:105-110.
10. Berlutti F, Pantanella F, Natalizi T, Frioni A, Paesano R, Polimeni A, Valenti P. Antiviral properties of lactoferrin -- a natural immunity molecule. Molecules. 2011; 16:6992-7018.
11. Fujimura T, Iguchi A, Sato A, Kagaya S, Hoshino T, Takeuchi T. The pain-relieving effects of lactoferrin on oxaliplatin-induced neuropathic pain. J Vet Med Sci. 2020; 82:1648-1654.
12. Ahmed HH, Essam RM, El-Yamany MF, Ahmed KA, El-Sahar AE. Unleashing lactoferrin's antidepressant potential through the PI3K/Akt/mTOR pathway in chronic restraint stress rats. Food Funct. 2023; 14:9265-9278.
13. Izumo N, Yukiko I, Kagaya N, Furukawa M, Iwasaki R, Sumino A, Hayamizu K, Nakano M, Hoshino T, Kurono H, Watanabe Y, Manabe T. Lactoferrin suppresses decreased locomotor activities by improving dopamine and serotonin release in the amygdala of ovariectomized rats. Curr Mol Pharmacol. 2021; 14:245-252.
14. Nagashima D, Mizukami N, Ogawa N, Suzuki S, Ohno M, Aoki R, Furukawa M, Izumo N. Bovine lactoferrin promotes neurite outgrowth in PC12 cells *via* the TrkA receptor. Int J Mol. 2024; 25:11249.
15. Aoki R, Ishido K, Furukawa M, Ishibashi Y, Nozaki S, Ito N, Toho M, Nagashima D, Izumo N. Bovine lactoferrin intake prevents hepatic injury in a mouse model of non-alcoholic steatohepatitis induced by choline and methionine deficiency. Drug Discov Ther. 2025; 19:230-236.
16. Ennaceur A. One-trial object recognition in rats and mice: methodological and theoretical issues. Behav Brain Res. 2010; 215:244-254.
17. Lueptow LM. Novel object recognition test for the investigation of learning and memory in mice. J Vis Exp. 2017; 30:55718.
18. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013; 48:452-458.
19. Mima Y, Izumo N, Chen JR, Yang SC, Furukawa M, Watanabe Y. Effects of *Coriandrum sativum* seed extract on aging-induced memory impairment in Samp8 mice. Nutrients. 2020; 12:455.
20. Bartus RT, Dean RT 3rd, Beer B, Lippa AS. The cholinergic hypothesis of geriatric memory dysfunction. Science. 1982; 217:408-414.
21. Falsafi SK, Deli A, Höger H, Pollak A, Lubec G. Scopolamine administration modulates muscarinic, nicotinic and NMDA receptor systems. PLoS One. 2012; 7:e32082.
22. Cheon SY, Koo BN, Kim SY, Kam EH, Nam J, Kim EJ. Scopolamine promotes neuroinflammation and delirium-like neuropsychiatric disorder in mice. Sci Rep. 2021; 11:8376.
23. Cutone A, Rosa L, Lepanto MS, Scotti MJ, Berlutti F, Bonaccorsi di Patti MC, Musci G, Valenti P. Lactoferrin efficiently counteracts the inflammation-induced changes of the iron homeostasis system in macrophages. Front Immunol. 2017; 8:705.
24. Guan S, Lu S, Zhang R, Wang Y, Yao X, Deng X, Lu J. Lactoferrin alleviates LPS-induced oxidative stress and necroptosis in liver by promoting mitophagy. J Agric Food Chem. 2025; 73:11948-11959.
25. Liu C, Peng Q, Wei L, Li Z, Zhang X, Wu Y, Wang J, Zheng X, Wen Y, Zheng R, Yan Q, Ye Q, Ma J. Deficiency of Lactoferrin aggravates lipopolysaccharide-induced acute inflammation *via* recruitment macrophage in mice. Biometals. 2023; 36:549-562.
26. Zhang Q, Li Y, Fan B, Wang F, Li Z, Pires Dias AC, Liu X, Wang Q. Dendrobium nobile Lindl ameliorates learning and memory deficits in scopolamine-treated mice. J Ethnopharmacol. 2024; 324:117416.
27. Wu SY, Pan BS, Tsai SF, Chiang YT, Huang BM, Mo

FE, Kuo YM. BDNF reverses aging-related microglial activation. J Neuroinflammation. 2020; 17:210.
28. Abdelhamid M, Jung CG, Zhou C, Abdullah M, Nakano M, Wakabayashi H, Abe F, Michikawa M. Dietary lactoferrin supplementation prevents memory impairment and reduces amyloid-β generation in J20 mice. J Alzheimers Dis. 2020; 74:245-259.
29. Zheng J, Xie Y, Li F, Zhou Y, Qi L, Liu L, Chen Z. Lactoferrin improves cognitive function and attenuates brain senescence in aged mice. J Funct Foods. 2020; 65:103736.

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***Brief Report***

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# Docosahexaenoic acid increases tyrosine hydroxylase phosphorylation at Ser40 without increasing tyrosine hydroxylase protein expression in differentiated NG108-15 cells

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**SUMMARY**: Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine biosynthesis, and its activity is regulated by its phosphorylation at specific serine (Ser) residues. In the present study, we investigated the effects of docosahexaenoic acid (DHA) supplementation on TH protein expression and phosphorylation at Ser31 and Ser40 during differentiation in the neuroblastoma–glioma hybrid cell line NG108-15. TH protein expression and phosphorylation levels were analyzed on day 0 (undifferentiated) and on days 5 and 6 (differentiated). Differentiation increased TH protein expression on days 5 and 6, and DHA supplementation did not affect these increases. Phosphorylation at Ser31 showed no significant change with differentiation. By contrast, phosphorylation at Ser40 exhibited a significant increase with increasing days of differentiation, and this was further augmented by DHA treatment. Collectively, these findings suggest that DHA enhances TH phosphorylation, particularly at Ser40, without affecting TH protein expression. DHA may therefore influence brain function not through changes in TH expression levels, but rather through the phosphorylation of TH at Ser40.

***Keywords***: DHA, n-3 polyunsaturated fatty acids, neuronal cell, tyrosine hydroxylase, tyrosine hydroxylase phosphorylation at Ser 40

## 1. Introduction

Tyrosine hydroxylase (TH) catalyzes the hydroxylation of tyrosine to L-3,4 dihydroxyphenylalanine and is the rate-limiting enzyme in catecholamine synthesis (*1,2*). The catecholamines dopamine, noradrenaline, and adrenaline play roles in many brain functions as well as in neuronal diseases and disorders (*3-5*).

TH contains serine (Ser) residues at Ser8, Ser19, Ser31, and Ser40 that can be phosphorylated by a variety of protein kinases. It is thought that the phosphorylation of Ser8 and Ser19 has no direct effect on TH enzymatic activity (*6,7*), whereas the phosphorylation of Ser31 and Ser40 directly regulates TH activity (*8*).

Polyunsaturated fatty acids are important constituents of mammalian phospholipids. Docosahexaenoic acid (DHA) is abundant in the central nervous system as a component of phospholipids and is reportedly involved in various brain functions including neuronal outgrowth, synaptic plasticity, mood regulation, learning, and memory (*9-14*). In the striatum of rats treated with 6-hydroxydopamine (6-OHDA), Ser40 phosphorylation and TH expression levels decrease; however, DHA treatment suppresses this decrease (*15*). By contrast, DHA does not alter Ser40 phosphorylation or TH expression in rats not treated with 6-OHDA (*15*).

NG108-15 cells are a neuroblastoma–glioma hybrid cell line that exhibit neuronal-like morphology and properties when differentiated (*16,17*). In the present study, we used NG108-15 cells to examine the effects of adding DHA to differentiation-inducing medium on TH protein expression and TH phosphorylation at Ser31 and Ser40.

## 2. Materials and Methods

### 2.1. Materials

NG108-15 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture medium and dexamethasone were purchased from Fujifilm Wako (Osaka, Japan). Dibutyryl cyclic adenosine monophosphate was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Nacalai

Tesque (Kyoto, Japan). Hypoxanthine, aminopterin, and thymidine (HAT) supplement (50×) was purchased from MP Biomedicals (Santa Ana, CA, USA). DHA was purchased from Cayman (Ann Arbor, MI, USA).

2.2. Cell culture

NG108-15 cells were grown and maintained in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% $CO_2$. Figure 1 shows the study design. Cells were seeded in 12-well plates at 5000 cells/cm$^2$. After 24 hours, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, HAT, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μM α-tocopherol, 0.2 mM dibutyryl cyclic adenosine monophosphate, and 100 nM dexamethasone, which was added to induce differentiation (*16,17*). Additionally, DHA (2 μM) bound to 0.05% fatty acid–free bovine serum albumin (BSA) was added to medium in the DHA(+) group. The medium for the DHA(−) group contained 0.05% fatty acid–free BSA without DHA. The cells were then cultured for 5 or 6 days.

2.3. Preparation of samples for western blot analysis

Cells were harvested in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $Na_2$ ethylenediaminetetraacetic acid, 1 mM egtazic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and the samples were sonicated. The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) using BSA as the standard (*18*).

Aliquots were then mixed with concentrated sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis sample buffer (final concentrations: 62.5 mM Tris-HCl, pH 6.8, 2% 2-mercaptoethanol, 10% glycerol, 2% SDS, and 0.01% bromophenol blue)).

2.4. Western blot analysis

For SDS polyacrylamide gel electrophoresis, samples containing equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels and subsequently transferred to polyvinylidene fluoride membranes (*19,20*). The membranes were blocked with polyvinylidene fluoride blocking reagent (Toyobo, Tokyo, Japan) before being incubated overnight at 4°C with the following primary antibodies: TH (#2792, Cell Signaling Technology, Danvers, MA, USA), phosphorylated-TH (Ser31) (#13041, Cell Signaling Technology), phosphorylated-TH (Ser40) (#2791, Cell Signaling Technology), and β-actin (A5441, Sigma). The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies (Dako, Glostrup, Denmark) and developed using SuperSignal West Pico (Thermo Fisher Scientific, Waltham, MA, USA) or ImmunoStar LD reagents (Fujifilm Wako). Signal detection and band intensity quantification were performed using an Amersham Imager 680 (Cytiva, Tokyo, Japan).

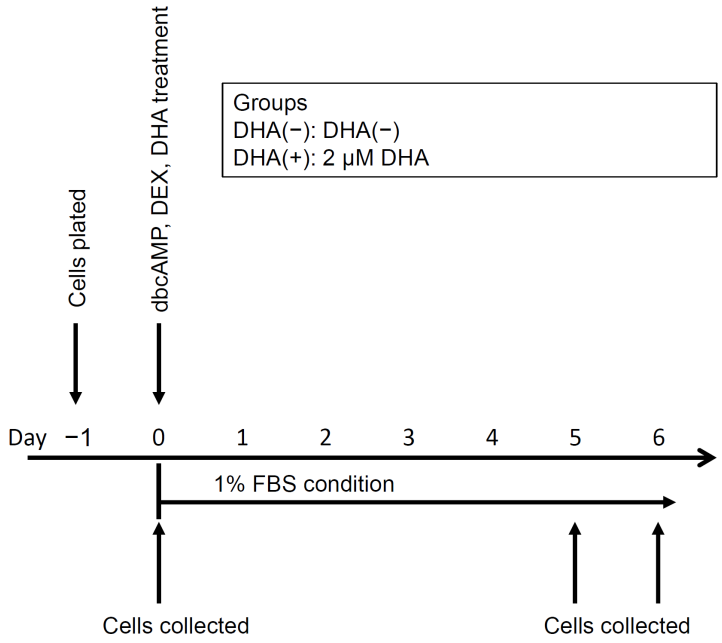


**Figure 1. Cell culture and treatment.** The day after seeding cells (day 0), differentiation medium (including dbcAMP and DEX) and/or DHA were added. On days 0, 5, and 6, cells were collected. dbcAMP, dibutyryl cyclic AMP; DEX, dexamethasone; DHA, docosahexaenoic acid.

2.5. Statistical analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$. Excel-Toukei software (2012, Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for the statistical analysis.

## 3. Results and Discussion

We investigated TH protein expression and phosphorylation on day 0 (undifferentiated) and days 5 and 6 (differentiated) to determine the effects of DHA on NG108-15 cells.

Figure 2 shows TH protein expression in NG108-15 cells after incubation for 5 or 6 days in differentiation-inducing medium. In the DHA(−) and DHA(+) groups, TH protein expression was increased with increasing days of differentiation. There was no significant difference between the groups with and without DHA addition.

Figure 3 shows TH phosphorylation at Ser31 in NG108-15 cells after incubation for 5 or 6 days in differentiation-inducing medium. There were no significant effects of days of differentiation, DHA treatment, or their interaction. However, in the DHA(+) group, a trend toward an increase was observed compared with the DHA(−) group.

Figure 4 shows TH phosphorylation at Ser40 in NG108-15 cells after incubation for 5 or 6 days in differentiation-inducing medium. TH phosphorylation at Ser40 was increased with increasing days of differentiation ($P < 0.005$), and the level was greater in the DHA(+) group than in the DHA(−) group. The level of TH phosphorylation at Ser40 in the DHA(+) group was increased by approximately 1.4-fold on day 5 and 2.2-fold on day 6 compared with the DHA(−) group.

It has been reported that TH is phosphorylated at Ser31 by both extracellular signal-regulated kinase1/2 and cyclin-dependent kinase 5 *in vitro* and *in vivo* (*21,22*). By contrast, TH is reportedly phosphorylated at Ser40 by cyclic AMP–dependent protein kinase (protein kinase A; PKA), protein kinase C, or protein kinase G *in vitro* and *in situ*, or by PKA *in vivo* (*21*). However, TH is reportedly dephosphorylated by protein phosphatase PP2A *in vitro* and *in vivo* (*23*). Additionally, *N*-docosahexaenoylethanolamine, a metabolite of DHA, is reported to be synthesized in the brain and activates PKA *via* GPR110 (*24*). In the present study, it is therefore possible that PKA was activated *via* this pathway, resulting in the increased phosphorylation of TH at Ser40.

Differentiation led to increased TH protein expression on days 5 and 6 compared with day 0. These increases were not affected by the addition of DHA (Figure

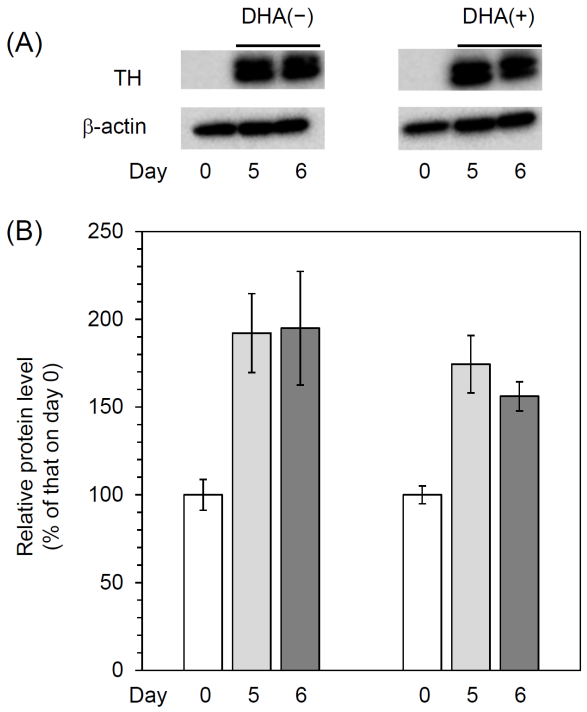


**Figure 2. Western blot analysis of TH protein levels in NG108-15 cells on days 0 (undifferentiated), 5, and 6 of differentiation.** (**A**) Representative western blots of TH and β-actin. (**B**) Semiquantitative analysis of TH/β-actin. Each column and bar represent the mean and standard error of the mean of four individual experiments. Using two-way ANOVA, there was an effect with days of differentiation ($P < 0.0005$). ANOVA, analysis of variance; DHA, docosahexaenoic acid; TH, tyrosine hydroxylase.

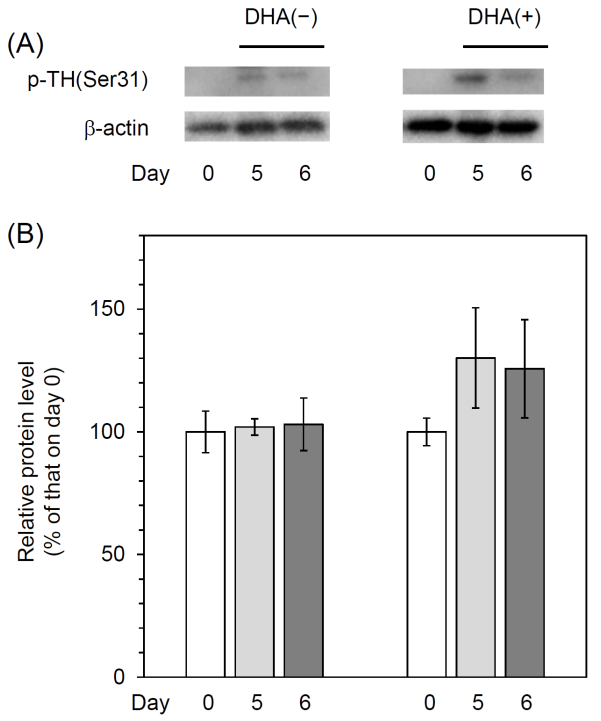


**Figure 3. Western blot analysis of TH with Ser31 phosphorylation in NG108-15 cells on days 0 (undifferentiated), 5, and 6 of differentiation.** (**A**) Representative western blots of TH with Ser31 phosphorylation and β-actin. (**B**) Semiquantitative analysis of TH with Ser31 phosphorylation/β-actin. Each column and bar represent the mean and standard error of the mean of four individual experiments. DHA, docosahexaenoic acid; p, phosphorylated; TH, tyrosine hydroxylase.

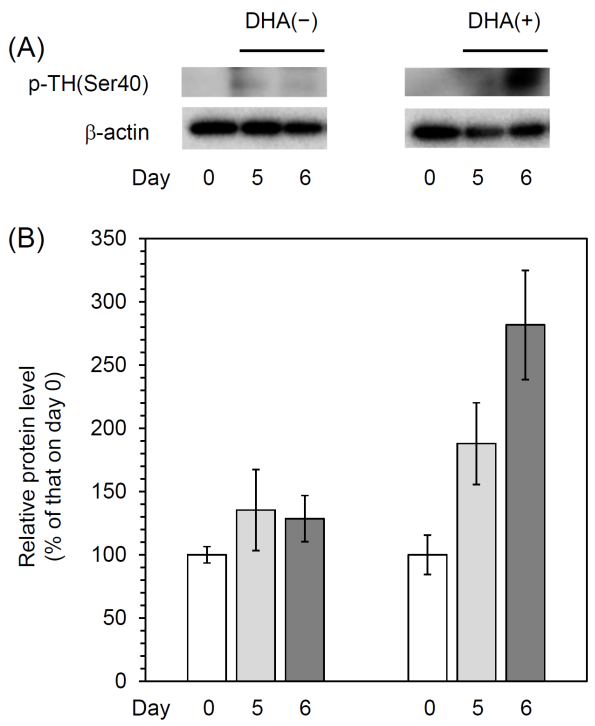


**Figure 4. Western blot analysis of TH with Ser40 phosphorylation in NG108-15 cells on days 0 (undifferentiated), 5, and 6 of differentiation.** (**A**) Representative western blots of TH with Ser40 phosphorylation and β-actin. (**B**) Semiquantitative analysis of TH with Ser40 phosphorylation/β-actin. Each column and bar represent the mean and standard error of the mean of four individual experiments. Using two-way ANOVA, there were effects with days of differentiation ($P < 0.005$) and DHA treatment ($P < 0.01$) and an interaction between days of differentiation and DHA treatment ($P < 0.05$). ANOVA, analysis of variance; DHA, docosahexaenoic acid; p, phosphorylated; TH, tyrosine hydroxylase.

2). Differentiation did not significantly increase TH phosphorylation at Ser31 on days 5 and 6 compared with day 0; however, a trend toward an increase was observed with the addition of DHA (Figure 3). Phosphorylation at Ser40 was increased with differentiation, and this was enhanced by the addition of DHA (Figure 4).

Under the conditions used in the present study, DHA did not affect TH protein levels; however, TH phosphorylation at Ser31 tended to increase, and TH phosphorylation at Ser40 increased significantly. Although our experiments were performed on cells *in vitro*, the addition of DHA into the medium increased the phosphorylation of TH at Ser40 in neuron-like differentiated NG108-15 cells. Previous studies have suggested that phosphorylation at Ser31 modulates phosphorylation at Ser40 (*25*). Moreover, phosphorylation at Ser40 is more directly involved in the activation of TH compared with the other phosphorylation sites (*8*).

In the striatum of Parkinson's disease model rats treated with 6-OHDA, Ser40 phosphorylation and TH expression levels decrease, and DHA treatment suppresses this decrease (*15*). However, this previous study did not examine Ser31 phosphorylation. Furthermore, DHA treatment in animals not treated with 6-OHDA does not increase Ser40 phosphorylation or TH expression levels (*15*). By contrast, our study indicates that Ser40 phosphorylation, which increases during differentiation induction, is further increased by DHA. Similarly, other studies have demonstrated that adding DHA to NG108-15 cells increases choline acetyltransferase expression, activity, and muscarinic receptors, although they did not examine TH protein expression or its phosphorylation (*16,17*).

In the present study, we examined the effects of DHA on TH expression and phosphorylation during the differentiation of NG108-15 cells. DHA did not alter TH protein levels; however, it increased TH phosphorylation, particularly at Ser40, which is a site that is directly linked to TH activation. Phosphorylation at Ser31 also showed an increasing trend. Together, these findings suggest that DHA may enhance TH activity through intracellular signaling pathways, thereby potentially promoting catecholamine synthesis. Decreased catecholamines are associated with various diseases and disorders including depression, attention deficit hyperactivity disorder, and pure autonomic failure (*3-5*). It has been reported that an increase in Ser40 phosphorylation, induced by phosphodiesterase inhibition and guanylate cyclase-C activation, leads to an improvement in motor deficits in a 6-OHDA-induced Parkinson's disease model (*26*). In summary, the present study provides evidence that DHA selectively promotes the Ser40 phosphorylation of TH without altering the total amount of TH protein in differentiated NG108-15 cells, thereby revealing a possible mechanism by which DHA modulates dopaminergic capacity. We believe that further studies may help to elucidate the relationship between DHA and these diseases and ultimately support their prevention or mitigation.

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**References**

1. Ikeda M, Fahien LA, Udenfriend S. A kinetic study of bovine adrenal tyrosine hydroxylase. J Biol Chem. 1966; 241:4452-4456.
2. Nagatsu T, Levitt M, Udenfriend S. Tyrosine Hydroxylase. The initial step in norepinephrine biosynthesis. J Biol Chem. 1964; 239:2910-2917.
3. Cho E, Yi JH, Jeon SJ, Kim DH, Kwon H, Jeon J, Kwon KJ, Jang DP, Moon M, Shin CY, Kim DH. Increases in

brain catecholamine levels counteract memory deficits and reduces Aβ deposition in 5XFAD male mice. Biomed Pharmacother. 2025; 193:118764.
4. Goldstein DS, Polinsky RJ, Garty M, Robertson D, Brown RT, Biaggioni I, Stull R, Kopin IJ. Patterns of plasma levels of catechols in neurogenic orthostatic hypotension. Ann Neurol. 1989; 26:558-563.
5. Lambert G, Johansson M, Agren H, Friberg P. Reduced brain norepinephrine and dopamine release in treatment-refractory depressive illness: evidence in support of the catecholamine hypothesis of mood disorders. Arch Gen Psychiatry. 2000; 57:787-793.
6. Haycock JW, Lew JY, Garcia-Espana A, Lee KY, Harada K, Meller E, Goldstein M. Role of serine-19 phosphorylation in regulating tyrosine hydroxylase studied with site- and phosphospecific antibodies and site-directed mutagenesis. J Neurochem. 1998; 71:1670-1675.
7. Sutherland C, Alterio J, Campbell DG, Le Bourdellès B, Mallet J, Haavik J, Cohen P. Phosphorylation and activation of human tyrosine hydroxylase *in vitro* by mitogen-activated protein (MAP) kinase and MAP-kinase-activated kinases 1 and 2. Eur J Biochem. 1993; 217:715-722.
8. Dunkley PR, Dickson PW. Tyrosine hydroxylase phosphorylation *in vivo*. J Neurochem. 2019; 149:706-728.
9. Fedorova I, Salem N, Jr. Omega-3 fatty acids and rodent behavior. Prostaglandins Leukot Essent Fatty Acids. 2006; 75:271-289.
10. Fujita S, Ikegaya Y, Nishikawa M, Nishiyama N, Matsuki N. Docosahexaenoic acid improves long-term potentiation attenuated by phospholipase A(2) inhibitor in rat hippocampal slices. Br J Pharmacol. 2001; 132:1417-1422.
11. Harauma A, Sagisaka T, Horii T, Watanabe Y, Moriguchi T. The influence of n-3 fatty acids on maternal behavior and brain monoamines in the perinatal period. Prostaglandins Leukot Essent Fatty Acids. 2016; 107:1-7.
12. Ikemoto A, Kobayashi T, Emoto K, Umeda M, Watanabe S, Okuyama H. Effects of docosahexaenoic and arachidonic acids on the synthesis and distribution of aminophospholipids during neuronal differentiation of PC12 cells. Arch Biochem Biophys. 1999; 364:67-74.
13. Ikemoto A, Kobayashi T, Watanabe S, Okuyama H. Membrane fatty acid modifications of PC12 cells by arachidonate or docosahexaenoate affect neurite outgrowth but not norepinephrine release. Neurochem Res. 1997; 22:671-678.
14. Ikemoto A, Ohishi M, Sato Y, Hata N, Misawa Y, Fujii Y, Okuyama H. Reversibility of n-3 fatty acid deficiency-induced alterations of learning behavior in the rat: level of n-6 fatty acids as another critical factor. J Lipid Res. 2001; 42:1655-1663.
15. Chitre NM, Wood BJ, Ray A, Moniri NH, Murnane KS. Docosahexaenoic acid protects motor function and increases dopamine synthesis in a rat model of Parkinson's disease *via* mechanisms associated with increased protein kinase activity in the striatum. Neuropharmacology. 2020; 167:107976.
16. Machová E, Málková B, Lisá V, Nováková J, Dolezal V. The increase of choline acetyltransferase activity by docosahexaenoic acid in NG108-15 cells grown in serum-free medium is independent of its effect on cell growth. Neurochem Res. 2006; 31:1239-1246.
17. Machová E, Nováková J, Lisá V, Dolezal V. Docosahexaenoic acid supports cell growth and expression of choline acetyltransferase and muscarinic receptors in NG108-15 cell line. J Mol Neurosci. 2006; 30:25-26.
18. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985; 150:76-85.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680-685.
20. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A. 1979; 76:4350-4354.
21. Dunkley PR, Bobrovskaya L, Graham ME, von Nagy-Felsobuki EI, Dickson PW. Tyrosine hydroxylase phosphorylation: regulation and consequences. J Neurochem. 2004; 91:1025-1043.
22. Bobrovskaya L, Damanhuri HA, Ong LK, Schneider JJ, Dickson PW, Dunkley PR, Goodchild AK. Signal transduction pathways and tyrosine hydroxylase regulation in the adrenal medulla following glucoprivation: an *in vivo* analysis. Neurochem Int. 2010; 57:162-167.
23. Ong LK, Page S, Briggs GD, Guan L, Dun MD, Verrills NM, Dunkley PR, Dickson PW. Peripheral Lipopolysaccharide Challenge Induces Long-Term Changes in Tyrosine Hydroxylase Regulation in the Adrenal Medulla. J Cell Biochem. 2017; 118:2096-2107.
24. Lee JW, Huang BX, Kwon H, Rashid MA, Kharebava G, Desai A, Patnaik S, Marugan J, Kim HY. Orphan GPR110 (ADGRF1) targeted by N-docosahexaenoylethanolamine in development of neurons and cognitive function. Nat Commun. 2016; 7:13123.
25. Stoop J, Douma EH, van der Vlag M, Smidt MP, van der Heide LP. Tyrosine hydroxylase phosphorylation is under the control of serine 40. J Neurochem. 2023; 167:376-393.
26. Douma EH, Stoop J, Lingl MVR, Smidt MP, van der Heide LP. Phosphodiesterase inhibition and Gucy2C activation enhance tyrosine hydroxylase Ser40 phosphorylation and improve 6-hydroxydopamine-induced motor deficits. Cell Biosci. 2024; 14:132.

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***Brief Report***

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# Analytical method validation and feasibility of salivary pregabalin measurement in Japanese volunteers: A pilot study

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**SUMMARY**: This pilot study evaluated whether salivary pregabalin concentrations reflect plasma levels in Japanese volunteers to support non-invasive therapeutic drug monitoring (TDM). Four healthy adults received a single 75-mg orally disintegrating tablet; unstimulated saliva and venous blood were collected 1 hour post-dose using a standardized passive-drool protocol with pre-collection rinsing. Pregabalin was quantified by a high-performance liquid chromatography with fluorescence detection. The assay met bioanalytical performance criteria across both matrices (excellent linearity, recovery > 94.2%, precision ≤ 10%, stability within 5.2%). At 1 hour, median concentrations were 1.96 μg/mL (plasma) and 0.466 μg/mL (saliva). In paired analysis ($n$ = 4), saliva and plasma showed a positive trend ($r$ = 0.838, $p$ = 0.298). Given the small sample size, these results are considered exploratory and demonstrate the feasibility of the analytical approach rather than providing definitive clinical evidence. Under standardized collection conditions, salivary pregabalin concentrations appear to yield clinically interpretable estimates of systemic exposure, warranting validation in larger, multi-time-point cohorts to establish actionable saliva-to-plasma conversion thresholds and evaluate clinical utility.

***Keywords***: Therapeutic drug monitoring, saliva, plasma, high-performance liquid chromatography, fluorescence detection, noninvasive sampling

## 1. Introduction

Pregabalin is a ligand of the α2δ subunit of voltage-gated calcium channels and attenuates excitatory neurotransmitter release. It is widely prescribed for neuropathic pain and fibromyalgia and is often considered among first-line options in chronic pain management (*1,2*). Its pharmacokinetic profile comprises rapid absorption (Time to maximum concentration: $T_{max}$ of approximately 1 h), negligible plasma protein binding (< 1%), and predominantly renal elimination, with most of the dose excreted unchanged in urine (*1,2*).

Older adults frequently experience age-related declines in renal function, increasing the risk of accumulation and central nervous system adverse effects such as dizziness and somnolence, which contribute to falls, fractures, and functional decline; because exposure is strongly influenced by renal function, these risks merit particular attention (*1,2*). Although initiation at low doses and gradual titration are recommended, inter-individual variability limits dose-based safety assessments alone.

Therapeutic drug monitoring (TDM) may therefore improve safety. However, blood-based TDM is invasive and difficult to implement in home-care or community settings. Saliva has emerged as a non-invasive alternative matrix: good saliva–plasma correlations have been reported for several drugs, including lithium and phenytoin, and recent reviews have synthesized broader evidence supporting saliva as a TDM matrix when the unbound fraction is clinically relevant (*3-5*).

Given its small molecular size and extremely low protein binding, pregabalin is mechanistically expected to diffuse into saliva (*2*). Nonetheless, human evidence remains limited and derives largely from Jordanian patients (*6*); no data have been reported in Asian populations. Generating preliminary data in Japanese individuals is therefore an essential first step toward evaluating whether salivary pregabalin concentrations can be used for monitoring in broader clinical settings. As an initial investigation, this pilot study focuses on feasibility and analytical behavior. We quantified salivary and plasma pregabalin concentrations in healthy Japanese volunteers and examined their relationship to assess the analytical feasibility of salivary pregabalin monitoring as

a non-invasive approach.

## 2. Materials and Methods

### 2.1. Study design

This pilot study assessed whether salivary pregabalin concentrations can serve as a surrogate for plasma levels. To coincide with the expected $T_{max}$, saliva and plasma were collected 1 hour post-dose (*1*). The protocol was approved by the Ethics Committee of Tokyo Metropolitan Bokutoh Hospital (Approval No. 05-112); written informed consent was obtained from all participants. This study was conducted in accordance with the Declaration of Helsinki (revised in 2013).

### 2.2. Participants and dosing

Four healthy adults (21-41 years; mean 27.5 ± 9.1 years; 2 males, 2 females) received a single 75-mg dose of pregabalin as orally disintegrating tablets (ODT). To mitigate oral-cavity contamination, participants avoided food and beverages (except water) for ≥ 1 h before dosing, swallowed without chewing, and rinsed the mouth with water immediately after complete disintegration (*7,8*). Saliva sampling was performed 60 min post-dose; a pre-collection water rinse was repeated 10 minutes before sampling.

### 2.3. Reagents and chemicals

Pregabalin, gabapentin (internal standard, IS), and 4-chloro-7-nitrobenzofurazan (NBD-Cl) were from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). A high-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, and distilled water were from Kanto Chemical Co., Inc. (Tokyo, Japan). Potassium dihydrogen phosphate ($KH_2PO_4$; FUJIFILM Wako, Osaka, Japan) was used in the mobile phase.

### 2.4. Analytical procedures

Pregabalin in plasma and saliva was quantified using a HPLC system (JASCO Corporation, Tokyo, Japan) consisting of a vacuum degasser, pump, gradient unit, and autosampler, with an FP-2020 spectrofluorometer (excitation/emission wavelengths [Ex/Em] of 470/530 nm). Separation was achieved on a C18 column (Capcell Pak C18 MG II, 250 × 4.6 mm, 5 µm; Osaka Soda Co., Ltd., Osaka, Japan) at 0.75 mL/min using 0.5% $KH_2PO_4$ (pH 4.5) and methanol. Samples (50 µL) were protein-precipitated, derivatized with NBD-Cl, and injected using gabapentin as the internal standard.

### 2.5. Sample preparation

Stock solutions of pregabalin and gabapentin (1 mg/mL each) were prepared in distilled water. To 50 µL of plasma or saliva, 10 µL of IS (0.2 µg gabapentin) and 140 µL of methanol were added. Following vortexing (60 s) and centrifugation (15,000× *g*, 10 min, 4°C), 100 µL of supernatant was mixed with 25 µL of borate buffer (0.25 mol/L, pH 10.5) and 100 µL of NBD-Cl (10 mg/mL). The mixture was reacted at 60°C for 15 min. A 20-µL aliquot was then injected.

### 2.6. Saliva collection

Unstimulated whole saliva was collected with participants seated and the head slightly tilted forward. After the standardized water rinse, saliva was collected by the passive drool method into polypropylene tubes for 3 min, targeting ≥ 1 mL.

#### 2.7.1. Calibration and method validation

Calibration ranges were 0.1, 0.5, 1, 5, 10, and 20 µg/mL for plasma and 0.0125, 0.025, 0.050, 0.10, 0.50, and 1.0 µg/mL for saliva. Method validation followed FDA bioanalytical guidelines (*9*). Recovery and accuracy were determined at 0.1-20 µg/mL (plasma) and 0.0125-1.0 µg/mL (saliva). Precision was evaluated using five sets of control samples intra-day and on five different days inter-day at 0.1, 0.5, 1, 5, 10, and 20 µg/mL (plasma) and 0.0125, 0.025, 0.050, 0.10, 0.50, and 1.0 µg/mL (saliva).

#### 2.7.2. Sample stability

Stability in plasma (0.1, 1.0, and 20 µg/mL) and saliva (0.0125, 0.10, and 1.0 µg/mL) was evaluated under bench-top (20°C, 6 h), processed sample (4°C, 24 h), long-term (−60°C, 4 weeks), and freeze-thaw (three cycles from −60°C) conditions ($n = 5$ for all conditions).

### 2.8. Statistical analysis

Pearson's correlation and Bland–Altman analyses were used to assess the plasma–saliva relationship, conducted with EZR (*10*) and JMP.

## 3. Results and Discussion

The assay demonstrated excellent linearity for plasma (0.1-20 µg/mL, $R^2 = 0.9994$) and saliva (0.0125-1.0 µg/mL, $R^2 = 0.9997$). Recovery exceeded 94.2%, with intra- and inter-day coefficient of variation (CV) s ≤ 10%. Stability tests under all conditions showed < 5.2% degradation, aligning with FDA bioanalytical guidance (*9*). While Idkaidek *et al*. (*6*) reported a TDM study in 44 Jordanian patients using capsules and proposed therapeutic ranges, the novelty of this study lies in its focus on the Japanese population using an ODT formulation and a standardized passive-drool collection protocol.

At 1 h after a single 75-mg oral dose, median concentrations were 1.96 µg/mL (CV 11.91%) in plasma and 0.466 µg/mL (CV 21.92%) in saliva (Table 1). Despite the risk of oral-cavity residue from the ODT formulation (*7,8*), the standardized passive-drool collection protocol was designed to mitigate potential contamination. The four paired observations showed a strong positive relationship (Pearson's $r = 0.838$) (Figure 1). However, this association did not reach statistical significance ($p = 0.298$), primarily due to the limited sample size ($n = 4$). These findings, along with the calculated saliva-to-plasma (S/P) ratios (median: 0.241; range: 0.185-0.275), suggest that salivary pregabalin concentrations may reflect systemic exposure, although confirmation in a larger cohort is required.

Bland–Altman analysis was performed for exploratory visualization (Figure 1). While all data points fell within the limits of agreement, these limits are highly unstable with $n = 4$ and should not be interpreted as a definitive demonstration of agreement. In Jordanian patients, trough and peak sampling at steady state showed good saliva–plasma correlations (0.71-0.83), and preliminary therapeutic ranges were proposed for both matrices, supporting feasibility while highlighting population characteristics, formulation (capsule vs ODT), and procedural differences as potential sources of variance (*6*).

Beyond pregabalin, strong saliva–serum relationships have been reported for lithium and for phenytoin; recent reviews underscore saliva's value as a non-invasive TDM matrix when the unbound fraction is clinically relevant (*3-5*). To enhance comparability across cohorts, future work should pre-specify formulation and sampling protocols (*e.g.*, capsule rather than ODT when feasible, standardized pre-rinse and timing).

From a clinical pharmacology perspective, pregabalin's pharmacokinetic profile renders exposure monitoring particularly relevant in individuals with impaired renal function. This profile also makes saliva—reflecting the diffusible, unbound fraction—mechanistically plausible as a surrogate matrix (*1,2*). However, older adults commonly exhibit reduced salivary flow, which may influence concentration measures; given our cohort's mean age (27 years), generalizability to elderly patients is limited (*11-13*). Future studies should incorporate multi-time-point paired sampling around and beyond $T_{max}$, capture salivary physiology (flow rate, pH) at collection and define clinically meaningful saliva–plasma thresholds for pregabalin TDM. Given the pilot nature of this investigation, the small sample size limits statistical power; accordingly, the observed

**Table 1. Plasma and salivary pregabalin concentrations in healthy volunteers**

| Volunteer No | Plasma (µg/mL) | Salivary (µg/mL) | S/P ratio |
|---|---|---|---|
| 1 | 1.94 | 0.359 | 0.185 |
| 2 | 2.23 | 0.551 | 0.247 |
| 3 | 1.66 | 0.389 | 0.234 |
| 4 | 1.98 | 0.544 | 0.275 |
| Median | 1.96 | 0.466 | 0.241 |
| (Range) | (1.66-2.23) | (0.359-0.551) | (0.185-0.275) |

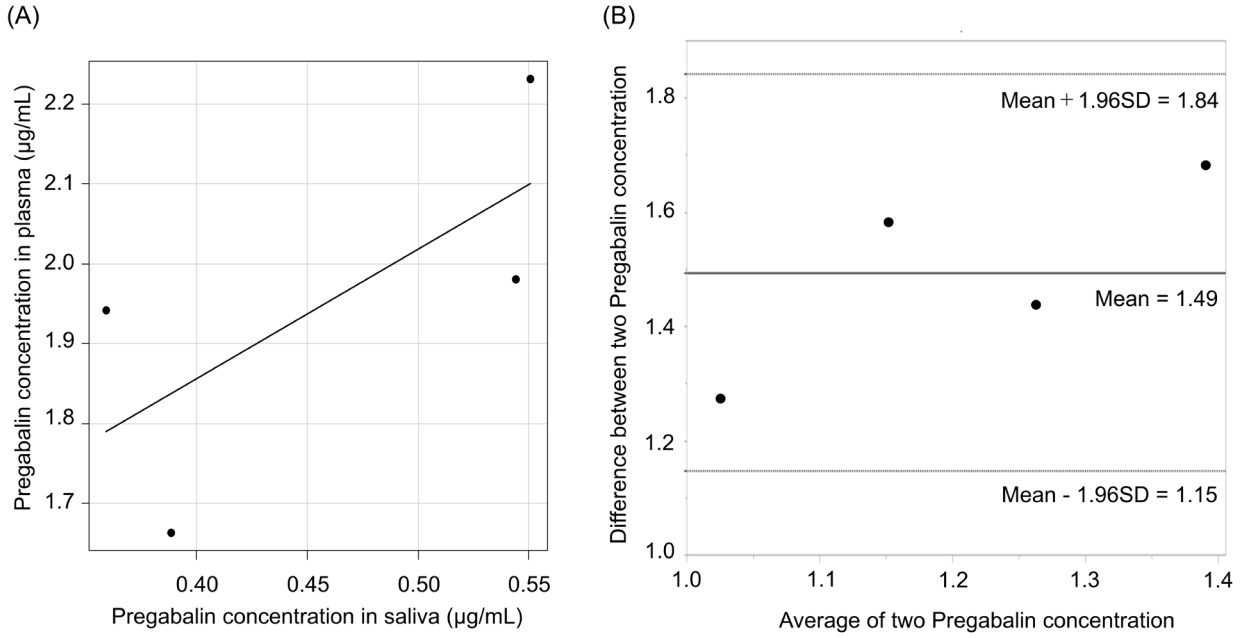


**Figure 1. Correlation between plasma and salivary pregabalin concentrations.** (**A**) Scatter plot showing the association between plasma and salivary pregabalin concentrations in healthy volunteers ($n = 4$), with Pearson's correlation coefficient indicating a positive relationship between the two matrices ($r = 0.838$). (**B**) Bland–Altman plot of paired plasma–saliva measurements ($n = 4$), showing the mean difference (1.49 µg/mL) and standard deviation (SD: 0.177 µg/mL), and the upper and lower limits of agreement (1.84 and 1.15 µg/mL), with all data points falling within the limits. This plot is intended for exploratory visualization of the differences between the two matrices.

correlation should be interpreted as exploratory rather than confirmatory. Nevertheless, the internal consistency of plasma concentrations across participants supports the feasibility of paired plasma–saliva assessment under a controlled protocol.

The lack of statistical significance ($p = 0.298$) and the potential for oral-cavity residue from the ODT formulation are the primary limitations of this feasibility study. The ODT formulation was selected to reflect its increasing use in Japanese clinical practice, particularly for patients with dysphagia. Although mouth rinsing was implemented to mitigate contamination, ODT residue remains a potential confounding factor. Future confirmatory studies should ideally use capsules to eliminate this risk or incorporate early post-dose rinse-fluid testing to verify the absence of residue.

These findings provide initial evidence that structured saliva collection can yield clinically interpretable pregabalin concentrations. Future studies should establish actionable saliva-to-plasma conversion thresholds using larger, multi–time-point cohorts— particularly in older adults and patients with renal impairment — to define decision-making targets and to assess clinical outcomes.

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**References**

1. Bockbrader HN, Radulovic LL, Posvar EL, Strand JC, Alvey CW, Busch JA, Randinitis EJ, Corrigan BW, Haig GM, Boyd RA, Wesche DL. Clinical pharmacokinetics of pregabalin in healthy volunteers. J Clin Pharmacol. 2010; 50:941-950.
2. Patsalos PN. Pregabalin. In: Antiseizure Medication Interactions. Springer, Cham, Switzerland, 2022; pp. 133-136.
3. Resztak M, Czyrski A, Sobiak J. Saliva as a matrix for therapeutic drug monitoring and disease biomarkers in children and adolescents. Pharmacol Rep. 2025; 77:921-961.
4. Parkin GM, McCarthy MJ, Thein SH, Piccerillo HL, Warikoo N, Granger DA, Thomas EA. Saliva testing to monitor therapeutic lithium levels: identification of clinical and environmental covariates and incorporation into a prediction model. Bipolar Disord. 2021; 23:679-688.
5. Rather MY, Farhat S, Rather MY. Use of saliva as an alternative matrix to serum/plasma for therapeutic drug monitoring using reverse-phase HPLC. Clin Ther. 2021; 43:2127-2135.
6. Idkaidek N, Hamadi S, El-Assi M, Al-Shalalfeh A, Al-Ghazawi A. Saliva versus plasma therapeutic drug monitoring of pregabalin in Jordanian patients. Drug Res (Stuttg). 2018; 68:596-600.
7. Klancke J, Gajendran J, Guillot A, Schichtel J, Tuereli A. Dissolution testing of orally disintegrating tablets. J Pharm Pharmacol. 2012; 64:911-918.
8. Almukainzi M, Araujo GLB, Löbenberg R. Orally disintegrating dosage forms. J Pharm Investig. 2018; 48:19-30.
9. U.S. Food and Drug Administration. Bioanalytical Method Validation: Guidance for Industry. May 2018. *https://www.fda.gov/media/70858/download* (accessed 15 January 2026)
10. Kanda Y. Investigation of the freely available easy-to-use software "EZR" for medical statistics. Bone Marrow Transplant. 2013; 48:452-458.
11. Xu F, Laguna L, Sarkar A. Ageing-related changes in quantity and quality of saliva: Where do we stand in our understanding? J Texture Stud. 2019; 50:27-35.
12. Vandenberghe-Descamps M, Labouré H, Prot A, Septier C, Tournier C, Feron G, Sulmont-Rosse C. Salivary flow decreases in healthy elderly people independently of dental status and drug intake. J Texture Stud. 2016; 47:353-360.
13. Morita I, Morioka H, Abe Y, Nomura T, Nakashima S, Sugiura I, Inagawa Y, Kondo Y, Kameyama C, Kondo K, Kobayashi N. Discordance between hyposalivation and xerostomia among community-dwelling older adults in Japan. PLoS One. 2023; 18:e0282740.

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# Associations between wound exudate amino acid profiles and microbial dissimilarity in wound and peri-wound skin in healing wounds

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**SUMMARY**: Dysbiotic wound microbiota, which is dissimilar to that of the peri-wound skin, delays healing in hard-to-heal wounds. However, rapid and practical methods for detecting wound dysbiosis are lacking and the characteristics of the wound environment in which dysbiosis occurs remain unclear. Consequently, microbiota-targeted care to prevent delayed healing has not yet been established. This study investigated the association between amino acid profiles in wound exudates and the degree of microbial dissimilarity between the wound and peri-wound skin microbiota. Nine wounds from eight patients receiving home care were analyzed. The concentrations of 18 amino acids were measured using high-performance liquid chromatography, and the microbiota were detected using 16S rRNA gene amplicon sequencing. The microbial dissimilarity was assessed using the weighted UniFrac dissimilarity index, and correlations between the relative abundances of amino acids, amino acid ratios, and microbial dissimilarities were evaluated using Spearman's rank correlation. Most of the wounds were in the healing phase. The relative abundance of arginine showed a strong correlation with microbial dissimilarity ($\rho = -0.80$, $p = 0.01$). Additionally, eight amino acid ratios (arginine/asparagine and arginine/tyrosine) were significantly correlated with microbial dissimilarity. These findings support the development of point-of-care tools for assessing wound microbiota and improving wound management.

***Keywords***: Commensal skin microbiota, wound microbiota, hard-to-heal wounds, wound fluid, wound healing

## 1. Introduction

Wound infection is characterized by bacterial proliferation within the wound bed, resulting in tissue damage and disruption of the healing process (*1*). Such infections impose a significant economic burden on healthcare systems, reduce patient quality of life (*2*), and increase the risk of mortality (*3*). The relationship between the host and bacteria in wounds is continuous and progresses through various stages of microbial presence, from contamination to colonization, local infection (covert and overt), and eventually to spreading and systemic infection (*4*). In particular, covert local wound infections are difficult to detect, as delayed healing often occurs before the appearance of overt signs and symptoms, resulting in delayed initiation of appropriate treatment. Therefore, early prevention of covert local wound infections is essential to promote timely wound healing.

The wound microbiota composition is associated with wound healing outcomes (*5*). In particular, dysbiosis, defined as the formation of wound microbiota that differs markedly from the patient's own peri-wound skin commensal microbiota, has been implicated in the development of covert local wound infections (*6*). Excessive inflammation and delayed wound healing have been observed in animal models that mimic dysbiotic wound microbiota without overt signs of infection. Furthermore, compared to wounds colonized by commensal skin microbiota, these models showed a reduced number of Forkhead box P3-positive cells, a marker of regulatory T cells (Tregs) with anti-inflammatory functions (*7*). Conversely, even when commensal skin microbiota are colonized, delayed healing is observed if Treg induction is suppressed (*8*). These findings suggest that dysbiosis induces covert

local wound infections by impairing Treg-mediated immune tolerance. Accordingly, the wound and peri-wound skin microbiota must be assessed in daily care, and interventions aimed at preventing or correcting dysbiosis must be implemented based on the assessment results. However, next-generation sequencing (NGS), which is commonly used to identify microbiota, is time-consuming and costly, making it impractical as a point-of-care diagnostic tool. Moreover, the characteristics of the wound environment during dysbiosis remain unclear.

Host-related factors, such as immune status, underlying diseases, and local wound characteristics, play major roles in the formation of wound microbiota (*9*). In the present study, we focused on amino acids in wound exudates as key components of the wound environment. Amino acids serve as essential nutrients for bacterial growth (*10*) and are known to vary according to wound conditions (*11-13*). Therefore, the amino acid profile of wound exudate may be associated with the composition of the wound microbiota. In the present study, we tested this hypothesis using wound exudates and microbiota samples obtained from patients with hard-to-heal wounds. Specifically, amino acid profiles were analyzed using high-performance liquid chromatography (HPLC), and the wound and peri-wound skin microbiota were identified using NGS. We then investigated the association between amino acid profiles and microbial dissimilarity in the wound and peri-wound skin in detail.

## 2. Materials and Methods

### 2.1. Ethical considerations

The study protocol was approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo (Approval No. 2022182NI-(3)) and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to their enrollment in the study.

### 2.2. Study design and participants

This prospective cohort study was conducted between January and March 2023. Participants were recruited from a home care clinic specializing in wound management that provides services in the Kanagawa and Tokyo prefectures, Japan. The inclusion criteria were as follows: individuals aged 20 years or above; those with hard-to-heal wounds extending into the subcutaneous tissue; and patients who received home care from the clinic and were followed up with a second visit 2 weeks later. Patients whose wounds were deemed difficult to swab according to the attending physician were excluded. Following baseline data collection, patients were followed-up until their next scheduled visit to assess their wound healing status. A complete enumeration method was used, which included all patients with hard-to-heal wounds who underwent home visits to the clinic during the study period.

### 2.3. Sample collection

To assess the microbiota, samples were collected by a trained wound researcher using flocked swabs (Puritan, Guilford, ME, USA) pre-soaked in sterilized saline containing 0.1% Tween-20 (Nacalai Tesque, Kyoto, Japan). To minimize contamination, both the wound bed and peri-wound skin were cleansed with a skin cleanser before sampling. Wound microbiota samples were obtained by swabbing a 1 $cm^2$ area at the center of the wound bed using Levine's technique (*14*). Peri-wound skin microbiota samples were collected from the surrounding skin on the cranial side of the wound (the area not covered by the wound dressing) using the Z-stroke technique, with the area swabbed twice for consistency (*15*). All swab samples were stored at −80°C until DNA extraction to preserve microbial integrity.

For amino acid analysis of the wound exudate, additional wound swab samples were collected using the same Levine technique, with flocked swabs soaked in 80 μL of sterilized saline (*16*). Exudate was extracted by centrifuging the swabs at 3,000× *g* for 1 min, and the supernatant was stored at −80°C until analysis.

### 2.4. Data collection

Wound healing status was evaluated by comparing the total DESIGN scores at baseline and follow-up (*17*). Wounds were classified as "deteriorated" if the DESIGN total score remained unchanged or increased, indicating worsening of the wound, and as "healing" if the total score decreased compared to baseline. Trained nurses independently evaluated the scores.

Wound images were captured using a digital camera (RX100II; Sony Corporation, Tokyo, Japan) combined with a color calibration chart (CASMATCH; Bear Medic Co., Tokyo, Japan) to ensure standardized image analysis. Patient demographic data (age, sex, physical function level, and underlying diseases) and wound-related data (type, location, duration, and treatment) were extracted from medical records.

### 2.5. Microbiome analysis

Bacterial DNA was extracted from swab samples using the QIAamp DNA Mini Kit (Qiagen N. V., Venlo, Netherlands) following previously described methods (*18*). The 16S rRNA gene amplicon sequencing was performed to characterize the wound and peri-wound skin microbiota. Polymerase chain reaction

amplification was conducted using a 16S Barcoding Kit 1-24 [SQK-16S024; Oxford Nanopore Technologies (ONT), Oxford, UK] targeting the near-full-length bacterial 16S rRNA gene. Sequencing was performed using a MinION portable sequencer (ONT) equipped with an FLO-MIN106D flow cell (ONT). Basecalling was conducted using the EPI2ME online platform (ONT), and microbial diversity analysis was performed using QIIME 2.

Microbiota composition was evaluated based on relative abundance, representing the proportion of each bacterium within the sample. To assess the microbial dissimilarity between the wound and peri-wound skin of the same individual, the weighted UniFrac dissimilarity index was calculated. This index ranged from 0 to 1, with higher values indicating greater dissimilarity in the microbiota.

### 2.6. Amino acid analysis

In this study, 18 amino acids were examined in the wound exudate. Sample preparation and HPLC analysis were performed according to previously described methods (*19*). The relative abundance of amino acids, defined as the ratio of their concentration to the total amino acid content, was calculated to evaluate the amino acid composition. In addition, the ratio of concentrations (amino acid ratio) was calculated for all combinations of the 18 amino acids.

### 2.7. Statistical analysis

Continuous variables are presented as median [interquartile range (IQR)], and categorical variables are presented as the number of cases (%). The Spearman's rank correlation coefficient was used to evaluate the association between the weighted UniFrac dissimilarity index in the wound and peri-wound skin microbiota and the relative abundance of individual amino acids and their ratios. To evaluate the influence of the repeated observation from one wound, a sensitivity analysis was performed by excluding one of the repeated measurements and recalculating the Spearman correlation coefficients using one observation per wound. All statistical analyses were performed using the EZR software (*20*). Statistical significance was set at $p < 0.05$.

## 3. Results and Discussion

Eight patients (eight wounds) were included in this study. Among the eight wounds, one wound was sampled at two time points separated by two weeks. Consequently, the analysis included nine observations derived from eight wounds. Patient and wound characteristics are summarized in Table 1. The median age of the participants was 74.5 years, and the median wound duration was 10 months. The DESIGN scores at baseline and two weeks later are shown in Table 2. The median change in the total DESIGN score over 2 weeks was −3 points (IQR: −4–−3). In eight out of nine cases (88.9%), the score decreased, and in the remaining case, the score remained unchanged. Thus, this cohort predominantly comprised wounds in the process of healing.

The composition of the wound and peri-wound skin microbiota is shown in Figure 1. The bacterial genus with the highest median relative abundance in the wound samples was *Staphylococcus* [median 2.8% (IQR: 0.7–64.4)]. In the peri-wound skin, *Staphylococcus* [median 36.6% (IQR: 6.7–61.5)] was also the predominant bacterial genus. The median weighted UniFrac dissimilarity index in the wound and peri-wound skin was 0.27 (IQR: 0.22–0.35). Similar findings have been reported in a previous study of pressure injuries showing a healing trend, in which both the weighted UniFrac dissimilarity index and the predominant bacterial genera were comparable to those observed in the present study (*6*). Together, these findings suggest that the microbiota observed in this cohort may represent a wound environment undergoing healing rather than dysbiosis.

**Table 1. Characteristics of participants and wounds ($n$ = 8)**

| | *n* or median | % or IQR |
|---|---|---|
| Age | 74.5 | (68.8–80.0) |
| Sex (male) | 4 | (50.0) |
| Degree of independence | | |
| Rank A (Independent, but may show occasional forgetfulness) | 2 | (25.0) |
| Rank B (Needs supervision or help with some daily tasks) | 3 | (37.5) |
| Rank C (Completely dependent on others for daily care) | 3 | (37.5) |
| Disease | | |
| Cardiovascular diseases | 4 | (50.0) |
| Endocrine disorders | 5 | (62.5) |
| Nervous diseases | 2 | (25.0) |
| Gastrointestinal diseases | 5 | (62.5) |
| Respiratory diseases | 2 | (25.0) |
| Braden scale | 18.5 | (10.0–20.8) |
| Treatment | | |
| Steroid drug | 3 | (37.5) |
| Dialysis | 2 | (25.0) |
| Wound area | | |
| Sacrum | 2 | (25.0) |
| Lower leg | 2 | (25.0) |
| Toe | 2 | (25.0) |
| Foot | 1 | (12.5) |
| Ischium | 1 | (12.5) |
| Wound type | | |
| Pressure injury | 3 | (37.5) |
| Amputation wound | 2 | (25.0) |
| Venous leg ulcer | 1 | (12.5) |
| Arterial ulcer | 1 | (12.5) |
| Skin ulcer in rheumatoid arthritis | 1 | (12.5) |
| Duration (month) | 10 | (4.5–18.0) |

IQR, interquartile range.

Correlation coefficients between the relative abundance of each amino acid and microbial dissimilarity were calculated (Figure 2A). Among the 18 amino acids, arginine showed a significant strong negative correlation ($\rho = -0.80$, $p = 0.01$). A higher relative abundance of arginine in the wound exudate was associated with increased microbial similarity between the wound and peri-wound skin. The biological significance of this association is supported by previous reports demonstrating that higher arginine concentrations are associated with skin microbiota profiles more closely resembling those of healthy individuals, even in patients with atopic dermatitis, in whom the pathogenic bacterium *Staphylococcus aureus* typically predominates (*21*). Furthermore, in non-inflammatory healthy skin, microbial composition has been linked to metabolites in the arginine biosynthesis pathway, suggesting that bacterial-derived arginine contributes to skin health (*22*). On the other hand, in infected chronic wounds, the concentrations of citrulline, ornithine, and arginase in the wound exudate were significantly higher than those in non-infected chronic wounds, despite the lack of a significant difference in arginine levels (*12*). Therefore, identifying biomarkers of microbial dissimilarity in infected wounds may require the examination of arginine as well as arginine metabolism.

Correlations between the ratios of all possible pairs among the 18 amino acids and the weighted UniFrac dissimilarity index were examined (Figure 2B). Significant correlations with microbial dissimilarity were observed for the following amino acid ratios (median [IQR]): arginine/histidine [$\rho = -0.76$, $p = 0.04$;

**Table 2. DESIGN score ($n$ = 9)**

| Components | Score | Baseline | Two weeks later |
|---|---|---|---|
| Depth | D3 | 5 (55.6) | 5 (55.6) |
| | D4 | 4 (44.4) | 4 (44.4) |
| Exudate | e1 | 0 (0.0) | 2 (22.2) |
| | e2 | 9 (100.0) | 7 (77.8) |
| Size | s1 | 3 (33.3) | 3 (33.3) |
| | s2 | 2 (22.2) | 2 (22.2) |
| | s3 | 1 (11.1) | 2 (22.2) |
| | s4 | 1 (11.1) | 1 (11.1) |
| | s5 | 1 (11.1) | 0 (0.0) |
| | S6 | 1 (11.1) | 1 (11.1) |
| Infection/inflammation | i0 | 2 (22.2) | 5 (55.6) |
| | i1 | 4 (44.4) | 4 (44.4) |
| | I2 | 3 (33.3) | 0 (0.0) |
| Granulation | g1 | 0 (0.0) | 5 (55.6) |
| | g2 | 4 (44.4) | 4 (44.4) |
| | G3 | 3 (33.3) | 0 (0.0) |
| | G4 | 2 (22.2) | 0 (0.0) |
| Necrotic tissue | n0 | 0 (0.00) | 3 (33.3) |
| | N1 | 8 (88.9) | 6 (66.7) |
| | N2 | 1 (11.1) | 0 (0.0) |
| Pocket | p0 | 9 (100.0) | 9 (100.0) |
| Total score | | 9 (8–13) | 7 (6–8) |

The number of wounds (%) per score is shown for each component of the DESIGN tool. The total score is also shown as the median (interquartile range) at each time point. Of the eight subjects, one patient (one wound) could be followed a total of two times every 2 weeks.

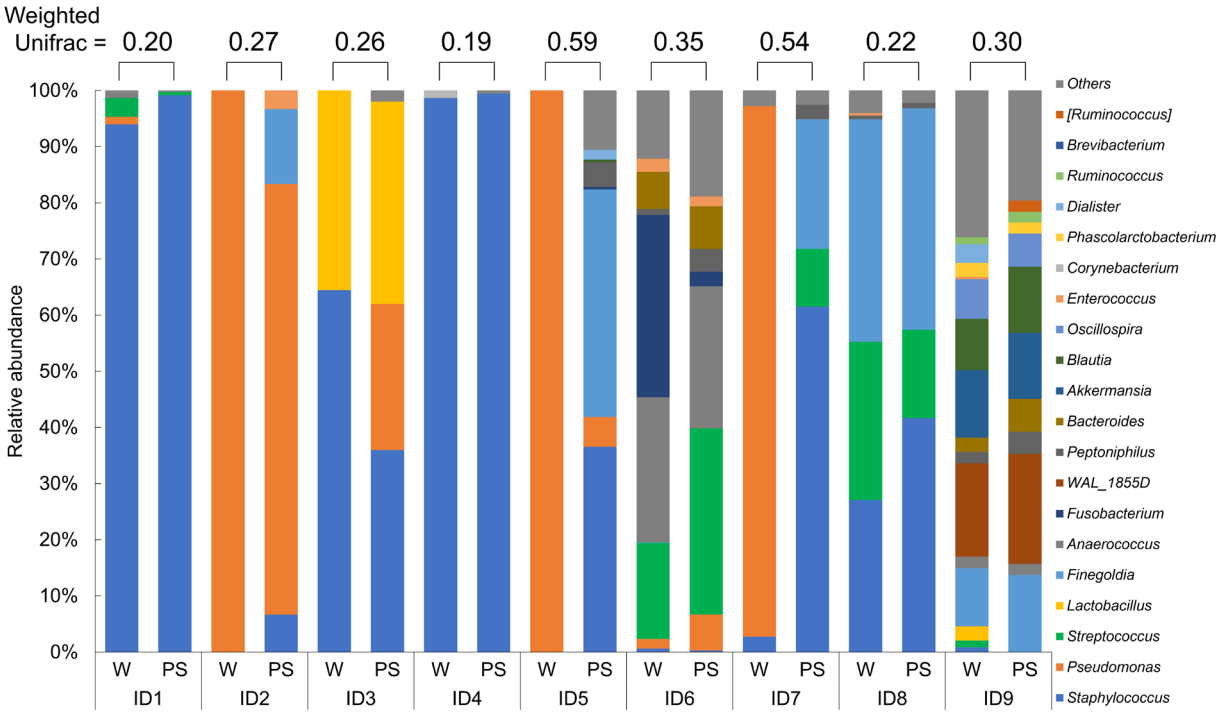
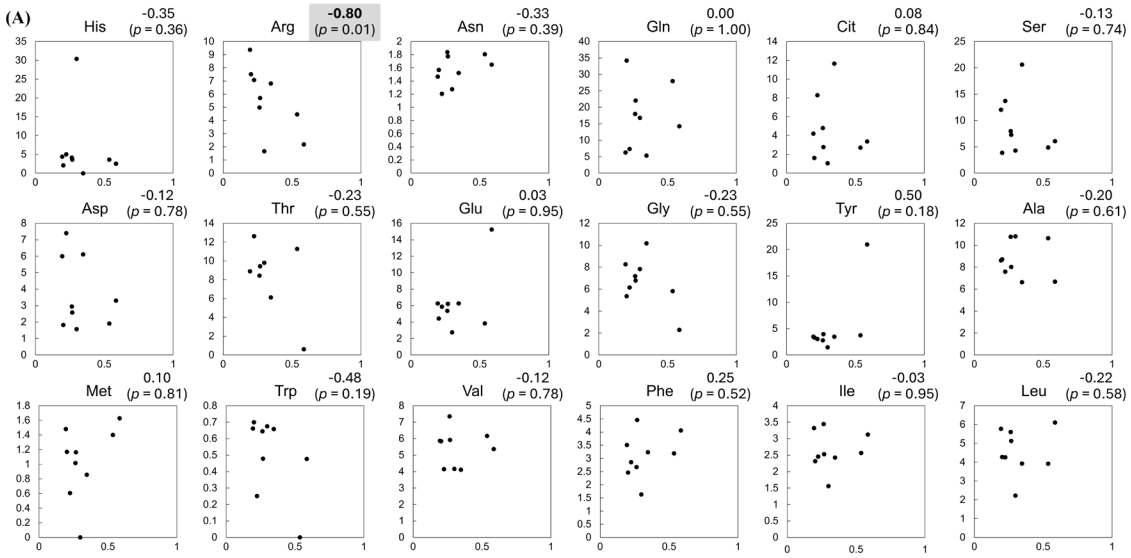



**Figure 1. Wound and peri-wound skin microbiota composition.** The top 20 bacterial genera present in the microbiota are shown. Bacterial genera with lower relative abundances were grouped under the category "Others." The relative abundance is represented in the vertical axis. Weighted UniFrac dissimilarity index values are shown at the top of the graph. W, wound samples; PS, peri-wound skin samples.




**(B)**

| ρ | His | Arg | Asn | Gln | Cit | Ser | Asp | Thr | Glu | Gly | Tyr | Ala | Met | Trp | Val | Phe | Ile | Leu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **His** | - | **-0.76** | 0.19 | 0.14 | -0.17 | -0.43 | -0.50 | -0.31 | -0.10 | -0.62 | 0.21 | 0.14 | 0.17 | -0.31 | 0.10 | 0.29 | 0.00 | 0.55 |
| **Arg** | 0.43 | - | **0.83** | 0.57 | 0.60 | 0.37 | 0.32 | -0.05 | **0.72** | 0.55 | **0.85** | 0.63 | 0.42 | 0.30 | 0.55 | **0.80** | **0.73** | 0.48 |
| **Asn** | -0.35 | **-0.83** | - | 0.05 | -0.07 | -0.13 | -0.28 | -0.38 | -0.07 | -0.48 | 0.03 | -0.45 | 0.02 | -0.37 | **-0.77** | 0.17 | -0.42 | -0.38 |
| **Gln** | -0.30 | -0.57 | -0.05 | - | -0.07 | -0.13 | -0.02 | -0.47 | 0.18 | -0.27 | 0.27 | -0.17 | -0.08 | -0.17 | -0.20 | 0.00 | -0.12 | -0.18 |
| **Cit** | -0.03 | -0.60 | 0.07 | 0.07 | - | -0.22 | -0.47 | -0.27 | 0.08 | -0.32 | 0.20 | -0.15 | 0.07 | -0.30 | 0.07 | 0.02 | 0.08 | 0.02 |
| **Ser** | 0.17 | -0.37 | 0.13 | 0.13 | 0.22 | - | -0.17 | -0.08 | 0.27 | -0.22 | 0.35 | 0.00 | 0.05 | -0.15 | 0.07 | 0.43 | 0.20 | 0.18 |
| **Asp** | 0.22 | -0.32 | 0.28 | 0.02 | 0.47 | 0.17 | - | -0.05 | 0.35 | 0.02 | 0.50 | 0.17 | 0.22 | -0.22 | 0.13 | 0.25 | 0.12 | 0.08 |
| **Thr** | 0.08 | 0.05 | 0.38 | 0.47 | 0.27 | 0.08 | 0.05 | - | 0.10 | 0.25 | 0.32 | 0.27 | 0.15 | 0.30 | 0.13 | 0.33 | 0.20 | 0.10 |
| **Glu** | -0.10 | **-0.72** | 0.07 | -0.18 | -0.08 | -0.27 | -0.35 | -0.10 | - | 0.18 | 0.62 | -0.17 | -0.23 | -0.52 | -0.17 | 0.00 | -0.37 | -0.40 |
| **Gly** | 0.33 | -0.55 | 0.48 | 0.27 | 0.32 | 0.22 | -0.02 | -0.25 | -0.18 | - | 0.35 | 0.50 | 0.17 | -0.02 | 0.28 | 0.37 | 0.12 | -0.17 |
| **Tyr** | -0.37 | **-0.85** | -0.03 | -0.27 | -0.20 | -0.35 | -0.50 | -0.32 | -0.62 | -0.35 | - | -0.27 | -0.43 | -0.48 | -0.48 | -0.35 | -0.63 | **-0.82** |
| **Ala** | -0.32 | -0.63 | 0.45 | 0.17 | 0.15 | 0.00 | -0.17 | -0.27 | 0.17 | -0.50 | 0.27 | - | 0.05 | -0.08 | 0.07 | 0.33 | -0.02 | 0.00 |
| **Met** | -0.45 | -0.62 | -0.07 | 0.05 | -0.17 | -0.17 | -0.36 | -0.21 | 0.21 | -0.29 | 0.45 | -0.14 | - | -0.45 | -0.33 | 0.10 | -0.48 | -0.24 |
| **Trp** | -0.17 | -0.69 | 0.17 | -0.12 | 0.10 | -0.10 | -0.02 | -0.67 | 0.33 | -0.24 | 0.29 | -0.21 | 0.00 | - | -0.21 | 0.00 | 0.05 | -0.12 |
| **Val** | -0.27 | -0.55 | **0.77** | 0.20 | -0.07 | -0.07 | -0.13 | -0.13 | 0.17 | -0.28 | 0.48 | -0.07 | 0.25 | -0.08 | - | 0.40 | 0.05 | -0.03 |
| **Phe** | -0.42 | **-0.80** | -0.17 | 0.00 | -0.02 | -0.43 | -0.25 | -0.33 | 0.00 | -0.37 | 0.35 | -0.33 | -0.12 | -0.25 | -0.40 | - | -0.60 | -0.47 |
| **Ile** | -0.20 | **-0.73** | 0.42 | 0.12 | -0.08 | -0.20 | -0.12 | -0.20 | 0.37 | -0.12 | 0.63 | 0.02 | 0.33 | -0.30 | -0.05 | 0.60 | - | -0.23 |
| **Leu** | -0.23 | -0.48 | 0.38 | 0.18 | -0.02 | -0.18 | -0.08 | -0.10 | 0.40 | 0.17 | **0.82** | 0.00 | 0.15 | -0.18 | 0.03 | 0.47 | 0.23 | - |

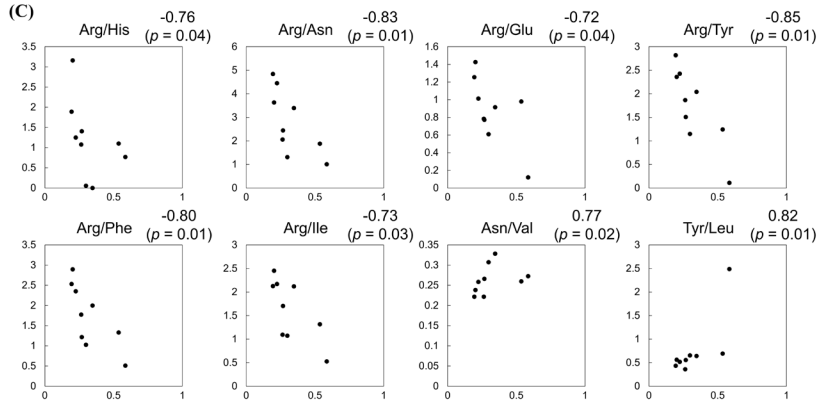



**Figure 2. Associations between wound exudate amino acid profiles and microbial dissimilarity. (A)** Scatter plots of the relative abundance of amino acids and the weighted UniFrac dissimilarity index. Data for each sample are plotted with the relative abundance of amino acids on the vertical axis and the weighted UniFrac dissimilarity index value on the horizontal axis. The numbers shown in the upper right of each scatter plot represent Spearman's rank correlation coefficients (p-value). Among the 18 amino acids, only arginine showed a statistically significant correlation ($\rho = -0.80$, $p = 0.01$). **(B)** Summary of Spearman's rank correlation coefficients between amino acid ratios derived from the 18 amino acids in wound exudate and the weighted UniFrac dissimilarity index. Amino acid ratios showing statistically significant correlations are highlighted in bold within shaded grey cells. Each amino acid ratio was calculated by dividing the concentration of the amino acid listed in the columns by that listed in the rows. For histidine (His), methionine (Met), and tryptophan (Trp), some wounds had concentrations of 0 mM, resulting in different correlation coefficients depending on whether these amino acids were used as the numerator or denominator of the ratio. **(C)** Scatter plots of amino acid ratios and microbial dissimilarities. The data for each sample are plotted with the amino acid ratio on the vertical axis and the weighted UniFrac dissimilarity index value on the horizontal axis. The numbers shown in the upper right of each scatter plot represent the correlation coefficients (p-value). Correlation coefficients shown in this figure are based on the primary analysis including nine observations derived from eight wounds. His, histidine; Arg, arginine; Asn, asparagine; Gln, glutamine; Cit, citrulline; Ser, serine; Asp, aspartic acid; Thr, threonine; Glu, glutamic acid; Gly, glycine; Tyr, tyrosine; Ala, alanine; Met, methionine; Trp, tryptophan; Val, valine; Phe, phenylalanine; Ile, isoleucine; Leu, leucine.

1.17 (1.00–1.53)], arginine/asparagine [ρ = −0.83, $p$ = 0.01; 2.44 (1.88–3.63)], arginine/glutamic acid [ρ = −0.72, $p$ = 0.04; 0.91 (0.77–1.01)], arginine/tyrosine [ρ = −0.85, $p$ = 0.01; 1.87 (1.24–2.36)], arginine/phenylalanine [ρ = −0.80, $p$ = 0.01; 1.77 (1.22–2.53)], arginine/isoleucine [ρ = −0.73, $p$ = 0.03; 1.70 (1.09–2.13)], asparagine/valine [ρ = −0.77, $p$ = 0.02; 0.26 (0.24–0.27)], and tyrosine/leucine [ρ = 0.82, $p$ = 0.01; 0.56 (0.52–0.66)] (Figure 2C). These findings suggest that specific amino acids and their concentration ratios in wound exudates may serve as potential biomarkers reflecting the degree of microbial similarity between the wound and peri-wound skin in healing wounds. Currently, microbiota identification is primarily performed using NGS techniques based on bacterial DNA sequencing (*23*). However, the substantial cost and time required for this method render it unsuitable for point-of-care testing, thereby limiting the integration of microbiota data into clinical decision-making for wound management. Therefore, future validation studies are warranted to establish these amino acids as clinically applicable biomarkers by evaluating their discriminative ability through receiver operating characteristic analysis and determining appropriate cutoff values based on sensitivity and specificity.

To evaluate the influence of the repeated observation, a sensitivity analysis was performed by excluding one of the repeated measurements and recalculating the Spearman correlation coefficients using one observation per wound ($n$ = 8). In this analysis, the correlation between arginine abundance and microbial dissimilarity was attenuated (ρ = −0.60, $p$ = 0.13) and was no longer statistically significant. In contrast, all eight amino acid ratios that were significantly associated with microbial dissimilarity in the primary analysis remained statistically significant. These findings suggest that amino acid ratios may represent more robust indicators of microbial dissimilarity than individual amino acids.

Among the significant amino acid ratios, the asparagine/valine and tyrosine/leucine ratios were low in most samples and exhibited a narrow range. In contrast, amino acid ratios with arginine as the numerator were generally higher. Together with the findings on arginine abundance, these results suggest that an arginine-rich wound environment may be associated with the formation or maintenance of a wound microbiota similar to that of the commensal skin microbiota.

Beyond its potential role as a microbiota-related biomarker, arginine may also influence the local immune environment. Arginine plays a role in immune tolerance mediated by Tregs. Tregs express arginase 2, which metabolizes arginine to enhance their suppressive capacity (*24*). These findings suggest that an arginine-rich microenvironment allows Tregs to function more effectively. Therefore, experimental studies using animal models are warranted to investigate the effects of arginine supplementation on wound microbiota and its local interaction with Tregs.

In conclusion, this study demonstrated that specific amino acids and their concentration ratios in wound exudates, particularly arginine, are associated with microbial similarity between the wound and peri-wound skin in wounds showing a trend toward healing. These findings suggest the potential utility of amino acid profiles as surrogate markers of wound microbiota dysbiosis. Given the limitations of the current sequencing-based microbiota analyses in clinical practice, amino acid–based biomarkers may serve as feasible alternatives for point-of-care wound assessment. Further studies involving larger cohorts and patients with delayed-healing wounds are warranted to validate their clinical applicability and specificity.

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**References**

1. Edwards R, Harding KG. Bacteria and wound healing. Curr Opin Infect Dis. 2004; 17:91-96.
2. Sharma A, Shankar R, Yadav AK, Pratap A, Ansari MA, Srivastava V. Burden of chronic nonhealing wounds: An overview of the worldwide humanistic and economic burden to the healthcare system. Int J Low Extrem Wounds. 2024; 25:371-378.
3. Ding X, Tang Q, Xu Z, Xu Y, Zhang H, Zheng D, Wang S, Tan Q, Maitz J, Maitz PK, Yin S, Wang Y, Chen J. Challenges and innovations in treating chronic and acute wound infections: from basic science to clinical practice. Burn Trauma. 2022; 10:tkac014.
4. Haesler E, Swanson T, Ousey K, Carville K. Clinical indicators of wound infection and biofilm: Reaching international consensus. J Wound Care. 2019; 28:S4-12.
5. Misic AM, Gardner SE, Grice EA. The wound microbiome: modern approaches to examining the role of microorganisms in impaired chronic wound healing. Adv wound care. 2014; 3:502-510.
6. Kunimitsu M, Nakagami G, Kitamura A, Minematsu T, Koudounas S, Ogai K, Sugama J, Takada C, Yeo S, Sanada H. Relationship between healing status and microbial dissimilarity in wound and peri-wound skin in pressure injuries. J Tissue Viability. 2023; 32:144-150.
7. Kunimitsu M, Nakagami G, Minematsu T, Koudounas S,

Sanada H. An *in vivo* critically colonised wound model with dysbiotic wound microbiota. Int Wound J. 2023; 20:648-658.
8. Kunimitsu M, Minematsu T, Koudounas S, Sanada H, Nakagami G. Relationship between dysbiotic wound microbiota and critical colonization: Involvement of FOXP3-positive cells in rats. Ann Plast Surg. 2024; 93:617-23.
9. Kunimitsu M, Kataoka Y, Nakagami G, Weller CD, Sanada H. Factors related to the composition and diversity of wound microbiota investigated using culture-independent molecular methods: a scoping review. Drug Discov Ther. 2021; 15:78-86.
10. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids. 2009; 37:1-17.
11. Berger MM, Binz P, Roux C, Charrière M, Scaletta C, Raffoul W, Applegate LA, Pantet O. Exudative glutamine losses contribute to high needs after burn injury. J Parenter Enter Nutr. 2022; 46:782-788.
12. Debats IBJG, Booi D, Deutz NEP, Buurman WA, Boeckx WD, van der Hulst RRWJ. Infected chronic wounds show different local and systemic arginine conversion compared with acute wounds. J Surg Res. 2006; 134:205-214.
13. Iizaka S, Sanada H, Minematsu T, Oba M, Nakagami G, Koyanagi H, Nagase T, Konya C, Sugama J. Do nutritional markers in wound fluid reflect pressure ulcer status? Wound Repair Regen. 2010; 18:31-37.
14. Levine NS, Lindberg RB, Mason AD, Pruitt BA. The quantitative swab culture and smear: A quick, simple method for determining the number of viable aerobic bacteria on open wounds. J Trauma. 1976; 16:89-94.
15. Rushing J. Obtaining a wound culture specimen. Nursing (Lond). 2007; 37:18.
16. Kunimitsu M, Nakagami G, Tunoda M, Akase T, Oe M. Investigation of the applicability of a sampling method of wound exudate using swabs for the amino acid analysis. J Nurs Sci Eng. 2023; 11:47-56.
17. Sanada, H, Moriguchi T, Miyachi Y, Ohura T, Nakajo T, Tokunaga K, Fukui M, Sugama J, Kitagawa A. Reliability and validity of DESIGN, a tool that classifies pressure ulcer severity and monitors healing. J Wound Care. 2004; 13:13-18.
18. Kunimitsu M, Nakagami G, Kitamura A, Minematsu T, Mugita Y, Ogai K, Sugama J, Aoki M, Takada C, Sanada H. Dissemination of microbiota between wounds and the beds of patients with pressure injuries: A cross-sectional study. Wound Pract Res. 2021; 29:70-76.
19. Tsunoda M, Ishida Y, Kunimitsu M. Amino acid analysis in rat wound exudate by high-performance liquid chromatography-fluorescence detection. Chromatography. 2024; 45:31-34.
20. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013; 48:452-458.
21. Tay ASL, Li C, Nandi T, *et al*. Atopic dermatitis microbiomes stratify into ecologic dermotypes enabling microbial virulence and disease severity. J Allergy Clin Immunol. 2021; 147:1329-1340.
22. Leung MHY, Tong X, Shen Z, Du S, Bastien P, Appenzeller BMR, Betts RJ, Mezzache S, Bourokba N, Cavusoglu N, Aguilar L, Misra N, Clavaud C, Lee PKH. Skin microbiome differentiates into distinct cutotypes with unique metabolic functions upon exposure to polycyclic aromatic hydrocarbons. Microbiome. 2023; 11:124.
23. Hodkinson BP, Grice EA. Next-generation sequencing: a review of technologies and tools for wound microbiome research. Adv Wound Care. 2015; 4:50-58.
24. Lowe MM, Boothby I, Clancy S, *et al*. Regulatory T cells use arginase 2 to enhance their metabolic fitness in tissues. JCI Insight. 2019; 4:e129756.

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DD&T
Drug Discoveries & Therapeutics

# Guide for Authors

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***Drug Discoveries & Therapeutics*** (Print ISSN 1881-7831, Online ISSN 1881-784X) welcomes contributions in all fields of pharmaceutical and therapeutic research such as medicinal chemistry, pharmacology, pharmaceutical analysis, pharmaceutics, pharmaceutical administration, and experimental and clinical studies of effects, mechanisms, or uses of various treatments. Studies in drug-related fields such as biology, biochemistry, physiology, microbiology, and immunology are also within the scope of this journal.

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