ISSN 1881-7831 Online ISSN 1881-784X

DD&T

Drug Discoveries & Therapeutics

Volume 20, Number 5
October 2026

IRCA BSSA
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www.ddtjournal.com

DD&T
Drug Discoveries & Therapeutics

ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

Drug Discoveries & Therapeutics is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group. It is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA.

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(*As of February 2026*)

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Editorial

DOI: 10.5582/ddt.2026.01068

The conversion window in hepatocellular carcinoma: When resectability alone is not an indication for surgery

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SUMMARY: Effective systemic therapy has expanded the possibility of resection in hepatocellular carcinoma, while making its timing more uncertain. Technical feasibility alone cannot establish whether surgery adds value to an effective treatment sequence. In this context, the conversion window can be understood as a patient-specific interval in which achievable tumor clearance, preserved hepatic and systemic reserve, and sufficient evidence of disease control are considered together. These factors should be distinguished from the multidisciplinary team's overall determination of whether surgery is likely to provide additional benefit. Evidence from a randomized study supports consolidation in selected patients but does not define an optimal date for surgery. Retrospective associations between treatment duration and pathological response remain vulnerable to selection bias and cannot justify universal timing thresholds. Serial assessment should incorporate remnant liver function, treatment toxicity, drug interruption, uncertainty regarding residual disease, and patient preferences. Future trials and prospective registries should evaluate explicit timing strategies from the point of initial surgical eligibility, including patients who subsequently become ineligible, using survival, safety, and quality-of-life outcomes to inform individualized decisions.

Keywords: immunotherapy, hepatic reserve, treatment sequencing, pathological response, shared decision-making

Modern systemic therapy has made curative-intent resection conceivable for patients with hepatocellular carcinoma (HCC) that was previously considered unsuitable for surgery. Atezolizumab plus bevacizumab, tremelimumab plus durvalumab, and nivolumab plus ipilimumab have expanded effective treatment options, although pivotal trials of those regimens did not establish when responding patients should undergo hepatectomy (*1-3*). Recent reviews have described this changing surgical landscape (*4,5*). Its central difficulty is increasingly temporal: once resection becomes feasible, should effective therapy be interrupted, continued to gauge disease behavior, or followed by another local strategy? Answering this question requires evidence of benefit from a treatment sequence, beyond evidence that surgery can be completed.

Terminological progress provides a foundation. Japanese oncological resectability criteria distinguish resectable and borderline categories after technical and functional feasibility have been considered (*6*). The Japan Liver Cancer Association subsequently distinguished functional from oncological conversion (*7*), while the European Association for the Study of the Liver (EASL) guidance and the Barcelona Clinic Liver Cancer (BCLC) 2026 update support a multidisciplinary assessment incorporating liver function, tumor characteristics, and patient priorities (*8,9*). These frameworks clarify eligibility but do not specify an optimal date for surgery for every patient. Resectability describes whether microscopically margin-negative (R0) surgery can be performed with adequate remnant function; an indication requires judging whether that surgery offers sufficient additional benefit over the available alternatives. These judgments should remain distinguishable throughout treatment.

The evidence now extends beyond retrospective enthusiasm. A systematic review of 38 studies involving 4,042 patients documented substantial variation in conversion rates across treatment strategies (*10*), and a multicenter hepatic arterial infusion chemotherapy (HAIC)-based cohort associated preoperative response with favorable postoperative outcomes (*11*). Neither finding isolates the contribution of resection. Zhao and colleagues' quantitative bias analysis rated the comparative evidence as having very low certainty and they indicated its vulnerability to surgical-eligibility confounding and time-related bias (*12*). Patients who are eligible for surgery have already survived induction, retained hepatic reserve, and avoided disqualifying progression; these advantages cannot automatically be

credited to surgery.

TALENTOP materially advances this discussion (*13*). Following induction atezolizumab plus bevacizumab, 201 of 489 enrolled patients were randomized to resection followed by systemic therapy or maintenance treatment. Median time to treatment failure was 20.4 versus 11.8 months (hazard ratio: 0.60; 95% confidence interval: 0.39-0.91). This supports a treatment strategy that includes surgery for a selected population, rather than another comparison between responders who underwent surgery and unselected nonsurgical patients. Nevertheless, the trial involved locally advanced disease with macrovascular invasion and no extrahepatic spread; overall survival remained immature, and severe treatment-related toxicity was more frequent with the surgical strategy. The trial compared treatment strategies at a selected post-induction decision-making point, not alternative dates for surgery. It therefore strengthens the case for appropriate consolidation while leaving the timing question open.

Building on response-guided consolidation proposals (*14*), the conversion window may be viewed as a patient-specific interval during which R0 resection is feasible, hepatic and systemic reserve are adequate, and observed disease control supports consideration of surgery, subject to comparison with continued treatment and other options. This is a proposed decision-making framework, not a validated score or a claim that every responder benefits from resection. Crucially, such an interval can exist without an established surgical indication: the expected incremental benefit must still justify operative morbidity, interruption of systemic control, and the patient's recovery burden. Nor must every patient pass through such an interval.

Time contributes information, but elapsed time alone does not establish favorable biology. Sustained control without new lesions may increase confidence that surgery is reasonable, whereas early shrinkage can coexist with occult dissemination. Conversely, a requirement for prolonged response could exclude patients whose anatomy and disease distribution already justify intervention. TALENTOP eligibility included partial response or stable disease after induction, underscoring the contention that objective shrinkage should not become an obligatory surgical gate (*13*). The useful question is what another treatment interval is expected to clarify or improve, and whether that information is worth the risk of becoming ineligible. Waiting without an explicit purpose or reassessment date is difficult to defend. Apparent stability also reflects the sensitivity of surveillance and the timing of scans. It offers probabilistic reassurance rather than a biological certificate, and prolonged observation may select favorable patients without improving any individual patient's outcome. This distinction matters when clinicians attribute success to the duration of induction itself.

Figure 1 separates three inputs for this judgment: technical feasibility, confidence in disease control, and hepatic and systemic reserve. Surgical opportunity is

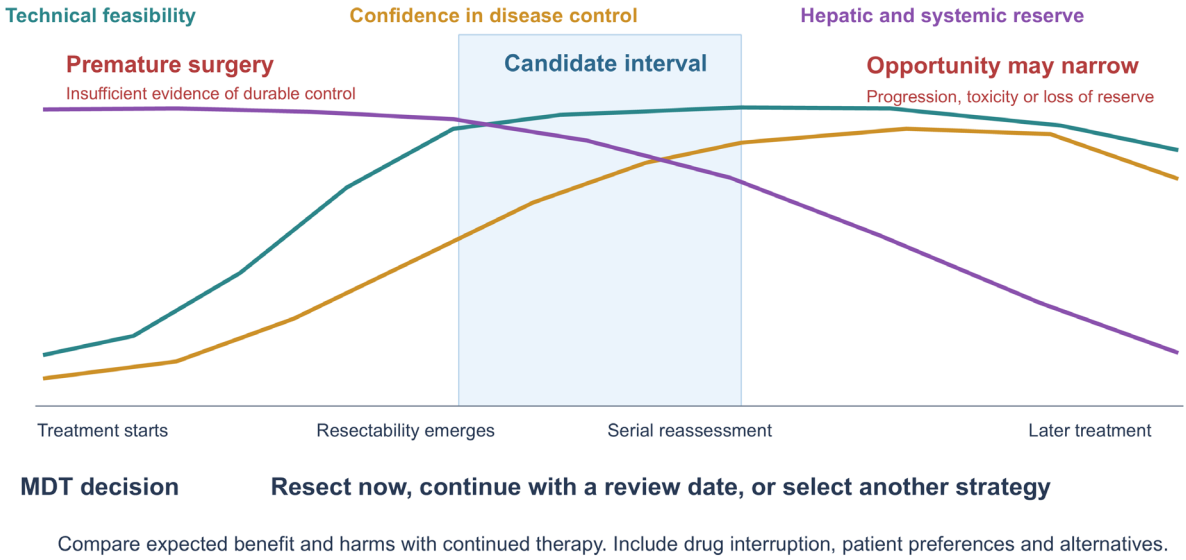


Figure 1. The conversion window in hepatocellular carcinoma. The schematic separates technical feasibility (teal), confidence in disease control (ochre), and hepatic and systemic reserve (purple) as inputs for a serial multidisciplinary team (MDT) assessment. The shaded candidate interval illustrates when these conditions may prompt consideration of resection; it does not itself establish an indication. The MDT compares expected surgical benefit and harm to continued treatment or alternative strategies, incorporating drug interruption and patient preferences. Premature intervention may not allow sufficient biological assessment, whereas progression, toxicity, or loss of reserve may narrow subsequent opportunities. Curves are qualitative illustrations, have no shared numerical scale, and do not represent patient data, validated thresholds, or a proven optimum. Reserve may remain stable or recover, and all trajectories depend on the patient and regimen. The framework is intended for prospective evaluation rather than prescription of a universal treatment duration.

the resulting assessment, rather than a fourth biological property. The schematic deliberately avoids numerical thresholds or a mathematically defined intersection. Technical feasibility may improve before disease behavior becomes sufficiently informative, while reserve may deteriorate with toxicity; however, reserve can also remain stable or improve after tumor control, antiviral treatment, or rehabilitation. Confidence may fall when new lesions appear. These trajectories are hypotheses for serial observation, not universal natural histories. Their paths should be expected to differ across HAIC, transarterial or radiation-based strategies, and systemic regimens (*5,15*).

An operational assessment should document what has changed since the previous multidisciplinary team (MDT) review. Imaging should address the distribution of viable disease, macrovascular involvement, new lesions, and the specific resection required, rather than tumor diameter alone. Response Evaluation Criteria in Solid Tumors (RECIST) and modified RECIST (mRECIST) findings should be interpreted alongside alpha-fetoprotein and des-gamma-carboxy prothrombin trends when informative; marker normalization is neither universally available nor proof of eradication. Child-Pugh status, albumin-bilirubin measures, portal hypertension, and future liver remnant volume and function should be reassessed with respect to the planned surgery, with indocyanine green testing where locally established (*8,15*). Frailty, nutritional status, treatment toxicity, and recovery expectations complete the assessment. A smaller tumor does not compensate for an unsafe remnant. The assessment should also retain the original reason for unresectability because improvement in tumor distribution, insufficient remnant function, and an unfavorable oncological profile represent different clinical problems. Patients initially eligible for resection who receive neoadjuvant therapy should not be pooled indiscriminately with those undergoing conversion. Otherwise, apparently similar treatment durations can describe fundamentally different decisions.

Background liver disease modifies these decisions through measured functional consequences, not through a geographical label. Viral suppression, cirrhosis severity, metabolic comorbidity, and prior liver-directed injury may alter the capacity to continue treatment or recover from hepatectomy (*8*). A worsening albumin-bilirubin trajectory should prompt investigation and reconsideration, but a single grade cannot define a universal closing date. Likewise, repeated local treatment should not be assumed to be inevitably harmful or automatically helpful. The MDT should explain whether the next intervention is intended to improve clearance, preserve parenchyma, or provide further evidence of disease control, and it should specify the finding that would trigger surgery or a change in strategy.

Perioperative drug management creates an additional interval within broader oncological decision-making. Bevacizumab prescribing information requires withholding treatment for at least 28 days before elective surgery and not resuming it for at least 28 days after major surgery and until adequate wound healing (*16*). This minimum is not a universal hepatectomy schedule; the extent of surgery, local protocol, and individual recovery may justify a longer interruption. Immune-related hepatitis and other unresolved toxicities require assessment rather than reliance on drug half-life alone (*15*). The MDT must anticipate disease behavior during treatment interruption and recovery, instead of declaring a window open on imaging and only addressing perioperative feasibility afterwards.

Recent timing data are useful precisely when their limitations remain evident. In a recent multicenter retrospective study, Li and colleagues analyzed 257 patients who underwent resection, stratified by treatment-to-surgery intervals of less than 12 weeks, 12-24 weeks, or more than 24 weeks (*17*). Major pathological response, defined using a study-derived residual viable tumor threshold of 15%, occurred in 41.3%, 66.2%, and 73.0% of patients, respectively. Although the authors favored 12-24 weeks, recurrence-free survival did not differ significantly across groups, and longer treatment did not clearly increase severe complications. These findings justify prospective evaluation; they neither prove a fixed optimum nor establish that later surgery is intrinsically unsafe. Patients who never survived to resection are particularly important to answering the question but were absent from this comparison.

Pathological response can support prognostic research without becoming a retrospective justification for a prospective decision. Another conversion cohort used a different major-response definition of at least 90% nonviable tumor, illustrating the need for pathological standardization (*18*). Both assessments require tissue obtained after surgery. Associations between pathology and outcome therefore cannot establish that delaying surgery to increase pathological response improves survival. Preoperative imaging and marker trajectories need to be validated with respect to clinically meaningful outcomes, including whether they identify a differential benefit from surgery. Circulating tumor DNA or tissue immune features may eventually add information, but they should not be compulsory gatekeepers while their value in decision-making remains unproven.

Clinical complete response presents a different uncertainty. Durable complete responses have been described after immunotherapy-based treatment (*19*), but absence of radiological activity cannot guarantee the absence of a viable tumor. In Aoki and colleagues' proof-of-concept study, only three of the 11 resected patients with a modified clinical complete response achieved a pathological complete response (*20*). Proposed "drug-off" (drug discontinuation) criteria included prior curative conversion and sustained marker normalization; they cannot be generalized into evidence that surgery may be

safely foregone after systemic therapy alone. Continued therapy, selected local consolidation, or structured observation may each be discussed, but a routine watch-and-wait policy lacks adequate comparative validation. The decision should reflect residual-disease uncertainty, the operative burden, and the patient's willingness to accept surveillance and possible retreatment.

These considerations make the MDT's conclusion more specific than "resectable." At each meaningful reassessment, it should record whether to proceed, continue treatment with a review date and explicit trigger, or pursue an alternative. The record should distinguish anatomical feasibility from the estimated benefit of acting now, identify the main uncertainty, and explain whether delay could resolve it. Patients should understand the plausible gains, the possibility of recurrence despite an R0 resection, the recovery period, and the potential need for subsequent therapy. Some will prioritize maximal local clearance; others will place greater weight on avoiding major surgery while control persists. Neither preference can be inferred from radiological response.

The next research step is to test dynamic strategies regarding the attainment of surgical eligibility. TALENTOP demonstrates the feasibility of randomization after induction, but it does not resolve early versus deferred resection across different regimens (*13*). Future trials should prespecify eligibility, induction exposure, reassessment intervals, permissible treatment interruptions, and the response or safety findings that trigger surgery. Overall survival, event-free outcomes, hepatic decompensation, perioperative morbidity, quality of life, and time without treatment would clarify the benefits and costs of the complete strategy. Pathological response should continue to be considered without displacing outcomes that matter to patients. A high resection rate alone cannot establish that a strategy is preferable. Comparative studies should also distinguish limited resections from major hepatectomy, since the same degree of tumor control can impose different operative costs. This would help determine whether preservation of future treatment options contributes to the value of consolidation.

Where randomization is impractical, prospective registries should identify all patients starting conversion treatment and a nested cohort at initial surgical eligibility. Treatment-to-resectability and resectability-to-surgery intervals should be reported separately, including missed opportunities and reasons for deferral. Target-trial emulation requires aligned eligibility, treatment assignment, and follow-up, with appropriate handling of treatment changes and time-varying confounding (*21*). Modeling surgery as a time-dependent exposure can address immortal time but cannot alone remove differences in evolving eligibility. The endpoint of interest is the outcome of a specified decision-making strategy among comparable patients, rather than survival conditional on eventually undergoing hepatectomy.

Dynamic resectability becomes clinically useful when it leads to a documented, revisable indication. The conversion window should make the tension between further biological information and ensuring safe surgery explicit, while avoiding the assumption that either immediate surgery or maximal response is invariably desirable. Modern therapy has expanded what surgeons can remove; evidence from a randomized study has now begun to clarify when adding resection can improve a treatment strategy. The remaining task is to determine for whom acting now offers more value than later reassessment, and how that judgment changes as tumor control and liver reserve evolve. Treating this as a prospective question will make the conversion window testable and help patients receive surgery when it is most likely to be of benefit.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received September 6, 2026; Accepted September 22, 2026.

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Released online in J-STAGE as advance publication September 25, 2026.

Original Article

DOI: 10.5582/ddt.2026.01057

Clinical investigation of effects of eldecalcitol on lipid profile and other metabolic parameters in osteoporosis-treatment-naïve postmenopausal women with osteoporosis

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SUMMARY: Osteoporosis is characterized by decreased bone mass and disruption of bone architecture. Eldecalcitol is the newest analog of activated vitamin D that can improve osteoporosis. In addition, dietary/activated vitamin D can improve metabolic parameters. Clinical studies have shown that activated vitamin D analogs improve glucose tolerance and hepatic function. However, the effects of activated vitamin D analogs, including eldecalcitol, on lipid parameters are yet to be clinically investigated. We aimed to reveal the effects of eldecalcitol on lipids and other metabolic parameters for 24 weeks in 54 osteoporosis-treatment-naïve postmenopausal women with osteoporosis. We measured metabolic parameters before and 24 weeks after administration of eldecalcitol (0.75 μg per day) without changing the patients' other medications. All patients completed the study without complications, and T-scores (lumbar spine and femoral neck) significantly improved from baseline to 24 weeks after eldecalcitol administration (each $P < 0.001$). Regarding metabolic parameters, low-density lipoprotein-cholesterol levels decreased significantly ($P = 0.007$), and levels of glycated hemoglobin, alanine aminotransferase, and γ-glutamyl transferase decreased significantly (each $P < 0.05$). Holm method for sensitivity analysis of the metabolic parameters revealed that there were no significant changes, but there was a trend of improvement in LDL-C ($P = 0.056$). Univariate regression analysis showed that age, body mass index, and changes in all metabolic parameters were not associated with T-score improvement. These results indicated that eldecalcitol might affect lipid metabolism and other metabolic parameters. Larger, controlled prospective studies with prespecified endpoints are required to determine whether eldecalcitol affects lipid and other metabolic parameters.

Keywords: Eldecalcitol, postmenopausal osteoporosis, lipid metabolism

1. Introduction

Osteoporosis is characterized by decreased bone mass and disruption of bone architecture, which increases the risk of fragility fractures (*1*). Therapeutic agents for osteoporosis have been recently developed (*2*), one of which is eldecalcitol (1α, 25-dihydroxy-2β-[3-hydroxypropoxy] vitamin D3), the newest analog of activated vitamin D (*3*). Previously, activated vitamin D analogs, as well as dietary vitamin D, have been reported to have efficacy on metabolic functions, such as glucose intolerance, lipid profiles, and hepatic function, both *in vitro* and *in vivo*, as well as clinically (*4-17*). Among them, a recent clinical study highlighted that eldecalcitol can improve glucose tolerance (*14*). Meanwhile, the association between lipid profiles and eldecalcitol remains unclear, as few studies have previously investigated this relationship *in vitro* and *in vivo* (*18*). Besides, the effects of eldecalcitol on lipid profiles have not been clinically investigated in the literature. Therefore, in this real-world study, we aimed to investigate the effects of eldecalcitol on lipid profiles and other metabolic parameters in postmenopausal women with osteoporosis.

2. Patients and Methods

2.1. Ethical approval of the study protocol

This study was approved as a retrospective study by the Ethics Review Committee of Nagasaki Prefecture Iki Hospital (Nagasaki, Japan; approval number: 2016-02) and conducted in accordance with the principles of the Declaration of Helsinki. All the participants provided written informed consent for inclusion

2.2. Study participants

We recruited 54 postmenopausal female individuals who were first diagnosed with osteoporosis at Nagasaki Prefecture Iki Hospital between November 2016 and October 2018, who satisfied the diagnosis of osteoporosis in accordance with widely used criteria (*19*); who did not have endocrine diseases that affect bone mass, such as hyperparathyroidism, Cushing's syndrome, pheochromocytoma, primary aldosteronism, thyroid dysfunction, and other diseases; and whose drugs, except for eldecalcitol, were not changed during the study period. The complications were defined. Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg and/or the use of antihypertensive drugs. Diabetes mellitus was defined as any combination of fasting plasma glucose (FPG) level ≥ 126 mg/dL, random blood sugar level ≥ 200 mg/dL, glycated hemoglobin (HbA1c) level ≥ 6.5%, or use of antidiabetic agents. Dyslipidemia was defined as any combination of total cholesterol level ≥ 220 mg/dL, low-density lipoprotein-cholesterol (LDL-C) level ≥ 140 mg/dL, high-density lipoprotein-cholesterol (HDL-C) level < 40 mg/dL; triglyceride (TG) level ≥ 150 mg/dL, or use of lipid-lowering drugs (*20*). Participant characteristics, including their medical treatment, are shown in Table 1. 27 participants (50.0%) were receiving antihypertensive treatment, 3 (5.6%) were receiving antidiabetic treatment, and 21 (38.9%) were receiving treatment for dyslipidemia. Regarding their medications for dyslipidemia, all patients who received treatment for dyslipidemia were medicated with a statin, and four patients received ezetimibe in addition to a statin. Medications for dyslipidemia, hypertension, and diabetes mellitus, as well as other drugs, were not changed during the study period.

2.3. Methods and disease definitions

To examine the effects of eldecalcitol (0.75 μg per day), treatment was administered and continued for 24 weeks. The following variables were measured at baseline and 24 weeks after administration of eldecalcitol: parameters of lipid metabolism (LDL-C, HDL-C, and TG), glycemic parameters (FPG, HbA1c), and hepatic enzymes (aspartate aminotransferase [AST], alanine aminotransferase [ALT], gamma-glutamyl transferase [γ-GTP]). Blood samples were collected after overnight fasting. LDL-C, HDL-C, TG, FPG, AST, ALT, and γ-GTP concentrations were investigated using the enzymatic method. HbA1c was determined using a high-performance chromatographic method. Regarding the effects of eldecalcitol on osteoporosis, T-scores (lumbar spine and femoral neck) were measured at baseline and 24 weeks after eldecalcitol administration with dual-energy X-ray absorptiometry (DXA; Discovery A, Hologic Inc., Waltham, MA).

2.4. Statistical analyses

Data are presented as mean ± standard deviation (SD). Changes in laboratory measurements from baseline to 24 weeks were assessed using a paired *t*-test and Holm method for sensitivity analysis for the metabolic parameters. The associations between changes in T-scores (lumbar spine and femoral neck) and metabolic parameters were examined using univariate regression analyses. *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed using Stata SE version 16 (StataCorp.2019. Stata Statistical Software, Release 16. College Station, TX: Stata Corp LLC.).

3. Results

The characteristics of the study participants are summarized in Table 1. All patients completed the study without any complications, including hypercalcemia and hypercalciuria. Regarding parameters of lipid metabolism, LDL-C and TG levels are decreased and HDL-C levels increased from baseline to 24 weeks after eldecalcitol administration; however, no statistical significance in a paired *t*-test was observed for HDL-C levels (LDL-C: 120.0 ± 24.5 *vs.* 113.9 ± 21.1 mg/dL, *P* = 0.007; HDL-C: 70.4 ± 15.1 *vs.* 71.2 ± 16.7 mg/dL, *P* = 0.137; TG: 111.7 ± 56.7 *vs.* 101.9 ± 49.8 mg/dL, *P* = 0.057).

Regarding glycemic parameters, HbA1c and FPG levels decreased from baseline to 24 weeks after eldecalcitol administration; however, no statistical significance in a paired *t*-test was observed for FPG levels (FPG: 100.5 ± 10.0 *vs.* 98.8 ± 9.2 mg/dL, *P* = 0.132; HbA1c: 5.84 ± 0.40 % *vs.* 5.78 ± 0.35 %, *P* = 0.047). Regarding hepatic enzymes, ALT, AST, and γ-GTP levels decreased from baseline to 24 weeks after eldecalcitol administration; however, no statistical significance in a paired *t*-test was observed for AST levels (AST: 21.7 ± 5.5 *vs.* 21.5 ± 4.6 U/L, *P* = 0.382; ALT: 17.4 ± 8.3 *vs.* 16.1 ± 5.5 U/L, *P* = 0.037; γ-GTP: 24.5 ± 20.0 *vs.* 21.3 ± 13.4 U/L, *P* = 0.032).

Holm method for sensitivity analysis for the metabolic parameters revealed that there were no significant changes from baseline to 24 weeks after eldecalcitol administration, but there was a trend of improvement in LDL-C (*P* = 0.056).

Regarding osteoporosis, the T-scores of the lumbar spine and femoral neck increased significantly from baseline to 24 weeks after administration of eldecalcitol (lumbar spine: − 1.97 ± 1.00 *vs.* − 1.84 ± 1.03, $P < 0.001$; femoral neck: − 2.67 ± 0.71 *vs.* − 2.48 ± 0.69, $P < 0.001$). All the changes in the parameters are shown in Table 2.

In addition, univariate regression analysis showed that age, body mass index, and improvements in all metabolic parameters were not associated with improvements in T-scores for both the lumbar spine and the femoral neck (Table 3).

Table 1. Characteristics of the patients in our study cohort

	n = 54
Age (years)	67.4 ± 7.2
BMI (kg/m^2)	22.3 ± 3.5
LDL-C (mg/dL)	120.0 ± 24.5
HDL-C (mg/dL)	70.4 ± 15.1
TG (mg/dL)	111.7 ± 56.7
FPG (mg/dL)	100.5 ± 10.0
HbA1c (%)	5.84 ± 0.40
AST (U/L)	21.7 ± 5.5
ALT (U/L)	17.4 ± 8.3
γ-GTP (U/L)	24.5 ± 20.0
P I NP (ng/mL)	45.4 ± 11.9
TRACP-5b (mU/dL)	495.6 ± 177.2
T-score (lumbar spine)	−1.97 ± 1.00
T-score (femoral neck)	−2.67 ± 0.71
Morbidity of hypertension	29 (53.7%)
Medical treatment for hypertension	27 (50.0%)
Administration of Ca blocker	24 (44.4%)
Administration of ACE inhibitor	3 (5.6%)
Administration of ARB	21 (38.9%)
Administration of beta-blocker	6 (11.1%)
Administration of thiazide derivative	3 (5.6%)
Morbidity of diabetes mellitus	10 (18.5%)
Medical treatment for diabetes mellitus	3 (5.6%)
Administration of DPP4 inhibitor	3 (5.6%)
Administration of biguanide	2 (3.7%)
Morbidity of dyslipidemia	29 (53.7%)
Medical treatment for dyslipidemia	21 (38.9%)
Administration of statin	21 (38.9%)
Administration of ezetimibe	4 (7.4%)

Data are presented as mean ± standard deviation (SD). ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; DPP4, dipeptidyl deptidase-4; FPG, fasting plasma glucose; γ-GTP, γ-glutamyl transferase; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; P I NP, type I procollagen N-terminal propeptide; SD, standard deviation; TG, triglyceride; TRACP-5b: tartrate-resistant acid phosphatase-5b.

4. Discussion

Eldecalcitol is the newest analog of activated vitamin D that can improve osteoporosis by facilitating bone formation and inhibiting bone resorption (*21*). The effects of eldecalcitol on osteoporosis have been revealed in clinical trials, and it has been approved for the treatment of osteoporosis (*22*). Previous *in vitro* and *in vivo* studies have demonstrated that dietary vitamin D can improve glucose tolerance, lipid profiles, and hepatic function (*4-6*). Clinical studies have also reported these efficacy (*6-9*). Previous *in vitro* and *in vivo* studies also showed that calcitriol, one of the activated vitamin D analogs, can improve glucose tolerance and hepatic function (*6,10,11*). In addition, the recent clinical study indicated that calcitriol improved hepatic dysfunction in patients with non-alcoholic fatty liver (*13*). Regarding eldecalcitol, Kawahara *et al.* reported that eldecalcitol can improve glucose tolerance and reduce the development of type 2 diabetes *via* both increased insulin secretion and reduced insulin resistance in a double-blind, multicenter, randomized, placebo-controlled study (*14*). In terms of hyperlipidemia, it was reported that activated vitamin D ameliorated AMPK/SIRT1 activity *in vitro* (*15*). Improvement of both AMPK and SIRT1 was known to improve hyperlipidemia (*16,17*). These results indicated that activated vitamin D analogs can improve lipid metabolism. However, the effect of eldecalcitol on lipid profiles remains unclear.

In the present study, regarding the glucose tolerance of the patients, a small decrease in HbA1c was observed. Regarding hepatic function, ALT and γ-GTP

Table 2. Metabolic parameters and T-scores at baseline and 24 weeks after eldecalcitol administration in the study cohort

	Baseline	24 weeks after	Mean paired change (95% CI)	*P*	*P* (Holm-adjusted)
LDL-C (mg/dL)	120.0 ± 24.5	113.9 ± 21.1	6.17 (1.33-11.00)	0.007*	0.056
HDL-C (mg/dL)	70.4 ± 15.1	71.2 ± 16.7	-1.26 (-3.55-1.03)	0.137	0.224
TG (mg/dL)	111.7 ± 56.7	101.9 ± 49.8	9.89 (-2.43-22.21)	0.057	0.228
FPG (mg/dL)	100.5 ± 10.0	98.8 ± 9.2	1.76 (-1.37-4.89)	0.132	0.528
HbA1c (%)	5.84 ± 0.40	5.78 ± 0.35	0.06 (-0.01-0.13)	0.047*	0.235
AST (U/L)	21.7 ± 5.5	21.5 ± 4.6	0.17 (-0.94-1.27)	0.382	0.382
ALT (U/L)	17.4 ± 8.3	16.1 ± 5.5	1.31 (-0.13-2.76)	0.037*	0.222
γ-GTP (U/L)	24.5 ± 20.0	21.3 ± 13.4	3.15 (-0.18-6.48)	0.032*	0.224
T-score (lumbar spine)	-1.97 ± 1.00	-1.84 ± 1.03	0.14 (0.08-0.20)	< 0.001*	-
T-score (femoral neck)	-2.67 ± 0.71	-2.48 ± 0.69	0.19 (0.10-0.29)	< 0.001*	-

Data are presented as mean ± SD (values at baseline and 24 weeks after). *P*-values were calculated using a paired *t*-test. P-values (Holm-adjusted) were calculated using the Holm method for sensitivity analysis of the metabolic parameters. $P < 0.05$ was considered significant (*). AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; CI, confidence interval; FPG, fasting plasma glucose; γ-GTP, γ-glutamyl transferase; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation; TG, triglyceride.

Table 3. Association between improvement in T-scores (lumbar spine and femoral neck) and improvement in metabolic parameter findings

Regression analysis	Improvement in T-score (lumbar spine)			Improvement in T-score (femoral neck)		
	β (SE)	95% CI	*P*	β (SE)	95% CI	*P*
Age	0.002 (0.004)	-0.006-0.011	0.624	0.002 (0.004)	-0.006-0.011	0.624
BMI	-0.000 (0.000)	-0.001-0.001	0.476	-0.000 (0.000)	-0.001-0.001	0.476
Improvement in LDL-C (mg/dL)	0.001 (0.002)	-0.003-0.004	0.669	0.001 (0.002)	-0.003-0.004	0.669
Improvement in HDL-C (mg/dL)	0.001 (0.004)	-0.006-0.008	0.796	0.001 (0.004)	-0.006-0.008	0.796
Improvement in TG (mg/dL)	-0.000 (0.001)	-0.001-0.001	0.907	-0.000 (0.001)	-0.001-0.001	0.907
Improvement in FPG (mg/dL)	-0.004 (0.003)	-0.009-0.001	0.149	-0.004 (0.003)	-0.009-0.001	0.149
Improvement in HbA1c (%)	-0.127 (0.657)	-0.356-0.102	0.271	-0.127 (0.657)	-0.356-0.102	0.271
Improvement in AST (U/L)	-0.008 (0.007)	-0.023-0.007	0.309	-0.008 (0.007)	-0.023-0.007	0.309
Improvement in ALT (U/L)	-0.000 (0.006)	-0.012-0.011	0.936	-0.000 (0.006)	-0.012-0.011	0.936
Improvement in γ-GTP (U/L)	0.001 (0.002)	-0.004-0.006	0.601	0.001 (0.002)	-0.004-0.006	0.601

$P < 0.05$ was considered significant. AST, aspartate aminotransferase; ALT, alanine aminotransferase; β, regression coefficient; BMI, body mass index; CI, confidence interval; FPG, fasting plasma glucose; γ-GTP, γ-glutamyl transferase; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SE, standard error; TG, triglyceride.

decreased during the treatment period. Regarding lipid metabolism, the levels of LDL-C decreased from before to 24 weeks after eldecalcitol administration. The change was significant by a paired *t*-test and was a trend by Holm method for sensitivity analysis. However, the clinical significance is uncertain. Statistical significance did not necessarily imply clinical relevance. In particular, although HbA1c showed a nominally significant decrease, the absolute mean change was only approximately 0.06% and its clinical significance is uncertain. Similar caution has been applied to the changes in the other metabolic parameters. Besides, the evaluation of glycemic parameters was performed only for the values of FPG and HbA1c, and that of liver function only for the values of AST/ALT/γ-GTP. Furthermore, changes in diet, physical activity, body weight, adherence to concomitant medications, regression to the mean, and other time-related factors were not investigated. They could have contributed to the observed changes. In addition, no statistically significant association was observed between changes in the measured metabolic parameters and changes in T-scores in the present study. Given the small sample size and exploratory nature of these analyses, the relationship between these changes remains uncertain and requires further investigation.

In this single-center study of 54 osteoporosis-treatment-naïve postmenopausal women with osteoporosis, small decreases in LDL-C, HbA1c, ALT, and γ-GTP were observed during 24 weeks of eldecalcitol treatment. However, these metabolic findings did not remain statistically significant after adjustment for multiple comparisons and should be considered exploratory. Because the present study was a single-center, single-arm retrospective study without a control group, a causal effect of eldecalcitol could not be established. Larger, controlled prospective studies with prespecified endpoints are required to determine whether eldecalcitol affects lipid and other metabolic parameters.

Acknowledgements

We thank Ms. Yumi Iriguchi for her assistance in conducting our study.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received August 17, 2026; Revised September 29, 2026; Accepted October 18, 2026.

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Released online in J-STAGE as advance publication October 21, 2026.

Original Article

DOI: 10.5582/ddt.2026.01013

HSD17B12 deficiency is associated with granulosa cell apoptosis and inflammatory transcriptomic signatures in experimental premature ovarian insufficiency models

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SUMMARY: Premature ovarian insufficiency (POI) remains a challenging condition with largely elusive etiology. Granulosa cell (GC) apoptosis is a key event in follicular atresia. This study investigates the correlation between 17β-hydroxysteroid dehydrogenase type 12 (HSD17B12) expression and ovarian dysfunction, specifically examining the transcriptomic landscape associated with *HSD17B12* modulation. A *Tripterygium wilfordii* polyglycoside suspension-induced POI mouse model was established to assess ovarian developmental status and *Hsd17b12* expression. HSD17B12 was overexpressed or knocked down in human granulosa tumor (KGN) and ovarian adenocarcinoma (SKOV3) cell lines. Transcriptomic sequencing was performed to analyze differential gene expression. Both mRNA and protein levels of *Hsd17b12* were significantly downregulated in the ovaries of the POI mouse model. *In vitro*, knockdown of *HSD17B12* suppressed cell proliferation and exacerbated apoptosis in both cell models. Transcriptomic analysis revealed that differentially expressed genes (DEGs) were predominantly enriched in immune regulation and inflammatory response pathways. Specifically, HSD17B12 deficiency led to the significant upregulation of genes involved in the "Cytokine-cytokine receptor interaction" and the "NF-κB signaling pathway". *HSD17B12* regulates proliferation and apoptosis in granulosa-like KGN cells, and *HSD17B12* deficiency is associated with an inflammatory transcriptomic signature in experimental POI models.

Keywords: Premature ovarian insufficiency, *HSD17B12*, granulosa cells, transcriptomics, NF-κB, inflammation

1. Introduction

Premature ovarian insufficiency (POI) is characterized by a reduced ovarian reserve in young women (< 40 years old), with a global prevalence estimated around 1% or higher in specific populations (*1*). Despite advances in genetic diagnosis and biomarker discovery, many POI cases remain idiopathic, underscoring the need to identify novel pathogenic factors and actionable molecular pathways (*2*). Two primary mechanisms are hypothesized to lead to POI: (1) insufficient acquisition of primordial follicles, typically occurring during the fetal period; and (2) excessive activation or accelerated atresia of primordial follicles. These processes lead to the irreversible depletion of the primordial follicle pool, ultimately diminishing follicle number and quality. Early diagnosis and intervention are crucial for POI; however, this remains challenging due to the insidious onset of the disease. Identifying pathogenic genes closely associated with POI progression may reveal potential therapeutic targets and provide a theoretical foundation for disease prevention, early diagnosis, and treatment.

Follicular depletion in POI is closely linked to granulosa cell (GC) dysfunction, where impaired proliferation and excessive apoptosis accelerate follicular atresia (*3,4*). Accumulating evidence also supports a role for ovarian inflammatory signaling in disrupting GC homeostasis. For example, theca-cell-derived IL 1β can act in a paracrine manner to activate NF κB signaling in GCs, increase oxidative stress, suppress steroidogenesis, and ultimately promote GC apoptosis (*5*). Consistently, NF κB-related inflammatory cascades are implicated in immune-mediated POI models and can be therapeutically modulated (*6*).

HSD17B12, a key enzyme catalyzing estrone-to-estradiol conversion, is broadly expressed in the ovary

and has been implicated in female fertility regulation (*7*). Importantly, human genetic and mouse model studies have demonstrated that HSD17B12 dosage insufficiency can directly cause POI and compromise ovarian reserve and fertility (*8*). However, the downstream transcriptomic programs linking *HSD17B12* deficiency to GC injury remain incompletely defined. In the present study, we established a *Tripterygium wilfordii* polyglycoside suspension-induced POI mouse model and manipulated *HSD17B12* expression in KGN cells, with SKOV3 used as a secondary ovarian epithelial context, to investigate its effects on GC survival and to characterize pathway-level changes, with a particular focus on inflammatory and NF-κB--associated signatures.

2. Materials and Methods

2.1. Establishment of the POI mouse model

Thirty-two female C57BL/6 mice (8–10 weeks old; Beijing Vital River Laboratory Animal Technology Co., Ltd.,Beijing,China) were housed in a specific-pathogen-free (SPF) barrier facility (20–24°C, 45%–65% humidity) at Inner Mongolia Medical University. They were provided with a standard diet, purified water, and a 12-hour light-dark cycle. All animal care procedures adhered to the ARRIVE guidelines and were approved by the Animal Ethics Committee of Inner Mongolia Medical University (Approval No. YKD202201213). Vaginal cytology was performed daily at 9:00 AM for 7 consecutive days. Mice with regular estrous cycles were randomly divided into two groups: a control group (n = 16) and a POI group (n = 16). The POI model was established by intragastric administration of a *Tripterygium wilfordii* polyglycoside suspension (Yuanda Medicine Huangshi Feiyun Pharmaceutical Co., Ltd., Huangshi, China.) (80 mg/kg/day) dissolved in 0.9% normal saline for 14 days. General physiological indexes and estrous cycles were monitored daily.

2.2. Tissue collection and processing

Two days post-treatment, mice were weighed, and orbital blood samples were collected. Samples were incubated at 4°C for 1 hour, and serum was obtained by centrifugation at 12,000 rpm for 15 min at 4°C, then stored at -80°C. Mice were sacrificed by cervical dislocation; uteri and ovaries were harvested and weighed. Right ovaries from four mice per group were fixed in 4% paraformaldehyde for histological analysis, while remaining tissues were snap-frozen for molecular studies.

2.3. Organ index, histological analysis, and hormone assay

2.3.1. Morphometric analysis of ovarian and uterine sections

Paraffin-embedded ovarian and uterine tissues were sectioned at 5 μm and stained with hematoxylin and eosin (H&E). Representative sections were imaged under a light microscope, and the cross-sectional areas of the ovary and uterus were measured using ImageJ software. For each animal, the mean area from serial sections was used for statistical analysis (mm^2).

2.3.2. Histological observation and follicle counting

Ovaries were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sections were cut at a thickness of 5 μm. To avoid double-counting, every section was mounted on glass slides, and the first section was discarded. Follicle counting was initiated from the third section and continued every 20 μm throughout the entire ovary. Under a light microscope, follicles were classified and counted as primordial follicles , early growth follicles, and atretic follicles based on their morphological characteristics.

2.3.3. Serum anti-Müllerian hormone (AMH) detection by ELISA

Mouse serum was collected and stored at -80°C. Before analysis, samples were thawed at room temperature and centrifuged at 12,000 × g for 5 min at 4°C to remove debris. The concentration of AMH in the serum was quantified using a commercial ELISA kit according to the manufacturer's instructions (Double-antibody sandwich method).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA (1 μg) from each sample was reverse-transcribed into cDNA using a MicroRNA RT Kit (Takara, Kusatsu, Japan). qRT-PCR was performed in triplicate on an ABI Prism 7300HT system using a SYBR Green PCR Kit and gene-specific primers (Takara, Table 1) according to the manufacturer's instructions. Relative mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blotting

Ovarian tissues were homogenized in RIPA lysis buffer (Beijing Suolaibao Technology Co., Ltd). Equal amounts of protein were separated by 8% SDS-PAGE

Table 1. Primer sequences

Gene	Sequence (5'-3')	Tm (°C)
HSD17B12-Forward	TCTGGAAATTCCCGACTTGGAC	68.14
HSD17B12-Reverse	TCTACCATGCCAGGCAGCAC	68.58
GAPDH- Forward	TGTGTCCGTCGTGGATCTGA	68.10
GAPDH- Reverse	TTGCTGTTGAAGTCGCAGGAG	67.68

(Coolaber) and subsequently transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in TBST and incubated overnight at 4°C with primary antibodies against HSD17B12 (1:500, BBI Life Sciences Corporation) or GAPDH (1:1,000, Abcam). Following washing, the membranes were incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (1:20,000, Santa Cruz Biotechnology) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) system.

2.6. Cell culture and lentiviral transduction

Human granulosa tumor (KGN) and ovarian adenocarcinoma (SKOV3) cells (Henan Province Engineering Research Center for Applied Microbiology, China) were used. Cells were infected with lentiviral vectors carrying HSD17B12 shRNA, HSD17B12 cDNA, or the corresponding negative control constructs. The HSD17B12 shRNA lentiviral construct was generated using the GV493 vector backbone, and the HSD17B12 overexpression construct was generated by cloning human HSD17B12 cDNA corresponding to NM_016142 into the GV492 vector backbone. Detailed information regarding the shRNA sequence, negative control sequence, overexpression plasmid, primer sequences, construct IDs, and viral titers is provided in Supplementary Table S1 (*https://www.ddtjournal.com/action/getSupplementalData.php?ID=321*).

Lentiviral transduction was performed using HiTransG P reagent for KGN cells and HiTransG A reagent for SKOV3 cells according to the manufacturer's instructions. Cells were seeded at $3 – 5 \times 10^4$ cells/mL (20-30% confluence) and infected at MOI = 100 (KGN) or MOI = 50 (SKOV3). Transduction efficiency (~80%) was verified by fluorescence microscopy 72 h post-infection. Stable cell lines were established and validated *via* qRT-PCR and Western blotting.

2.7. Cell proliferation assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Dalian Boglin Biotechnology). Transfected cells were seeded in 96-well plates and cultured for 0, 24, 48, and 72 h. At each time point, 10 μL of CCK-8 solution was added, and plates were incubated for 2 h. Absorbance at 450 nm was measured using a microplate reader. Experiments were performed in triplicate.

2.8. Flow cytometry analysis

Cells from each group were collected, washed with PBS (Meilunbio), and resuspended in 1 × binding buffer to a concentration of 1×10^6 cells/mL. Annexin V-PE (5 μL) and 7-AAD (5-10 μL) were added, mixed gently, and incubated at room temperature in the dark for 15 min. The above experiments were repeated three times. For each group, three independent events were captured, totaling 18 events. Apoptosis was then analyzed using flow cytometry (FACSCelesta/BD).

2.9. RNA sequencing and bioinformatics analysis

Total RNA was extracted from six experimental groups (KGN-NC, KGN-sh, KGN-OE, SKOV3-NC, SKOV3-sh, SKOV3-OE; n = 3 per group). Library construction and sequencing (NovaSeq X Plus, PE150) were conducted by Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. Raw reads were processed with fastp, aligned to the reference genome using HISAT2, and assembled with StringTie. Differential expression analysis was performed using DESeq2. Genes with $|\log_2 FC| \geqq 2$ and FDR < 0.05 were considered significantly differentially expressed (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted using Goatools and Python scipy, respectively (Bonferroni-corrected $P < 0.05$).

2.10. Statistical analysis

Data are presented as mean ± SD. Differences between two groups were analyzed using Student's *t*-test or Mann-Whitney *U* test where appropriate. Multiple comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. A two-tailed $P < 0.05$ was considered statistically significant.

3. Results

3.1. Phenotypic characterization of the POI Mouse Model

Intragastric administration of *Tripterygium wilfordii* polyglycoside suspension successfully induced ovarian dysfunction. Pre-treatment baseline characteristics showed no significant differences between groups. Post-treatment, the POI group exhibited a significant reduction in body weight compared to controls ($P < 0.001$, Table 2). Morphometric analysis revealed a marked decrease in ovarian area in the POI group ($P = 0.005$, Table 3), while uterine area showed a non-significant downward trend ($P = 0.386$, Table 3).

To validate the specific ovarian injury in the context of systemic toxicity, we performed a comprehensive phenotypic characterization of the model. While the model group exhibited significant weight loss compared to the control group, indicative of systemic effects, the ovaries showed specific pathological changes distinct from general toxicity. Histological analysis revealed a significant increase in the atretic follicle ratio ($P = 0.021$) and a decrease in the number of primordial ($P = 0.031$) and early growth follicles ($P = 0.021$) in the model group (Figure 1). Furthermore, serum AMH levels, a specific

marker of ovarian reserve, were significantly reduced (P = 0.002), and vaginal smears indicated disrupted estrous cycles (Figure 2). These findings confirm that the *Tripterygium wilfordii* polyglycoside suspension-induced model successfully recapitulates the specific ovarian dysfunction characteristic of POI, despite the presence of systemic weight loss.

3.2. Downregulation of HSD17B12 in POI ovaries and cell models

Given the critical role of *HSD17B12* in gonadal development, we assessed its expression levels. Both mRNA and protein levels of *Hsd17b12* were significantly downregulated in the ovaries of POI

Table 2. Body weight comparison between control and POI model mice (mean ± SD, *n* = 16)

Group	Control	POI	t/Z	*P*
Before modeling (body weight)	21.663 ± 0.344	21.531 ± 0.367	0.261	0.694
After modeling (body weight)	21.438 ± 0.336	19.069 ± 0.256	-3.663	< 0.001

Table 3. Ovarian and uterine morphometric analysis in control and POI mice (mean ± SD, subgroup for histology, *n* = 4 per group)

Group	Control (mm^2)	POI (mm^2)	t/Z	*P*
After modeling (ovary area)	3.617 ± 0.230	2.434 ± 0.199	-2.836	0.005
After modeling (uterus area)	83.238 ± 4.594	76.886 ± 6.730	0.779	0.386

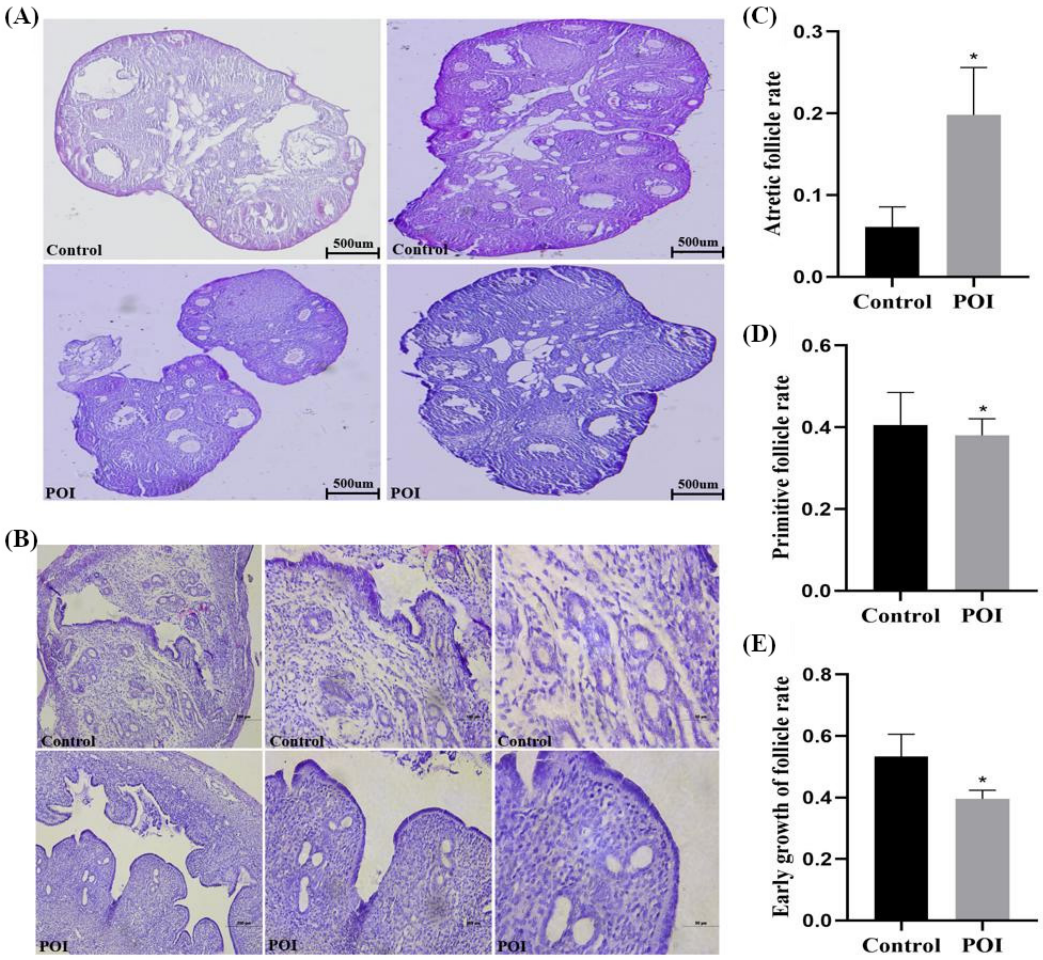


Figure 1. Histopathological analysis and follicle quantification in mouse ovaries. (**A**) Representative H&E staining of whole ovarian sections from Control and POI groups (Scale bar: 500 μm). (**B**) High-magnification views showing follicular morphology (Scale bar: 50 μm). (**C–E**) Quantification of the rates of (**C**) atretic follicles, (**D**) primordial follicles, and (**E**) early growth follicles (primary and secondary) in Control *vs.* POI ovaries (n = 4 mice per group). Data are presented as mean ± SEM. Statistical significance was determined by Student's t-test. *P < 0.05 *vs.* Control group.

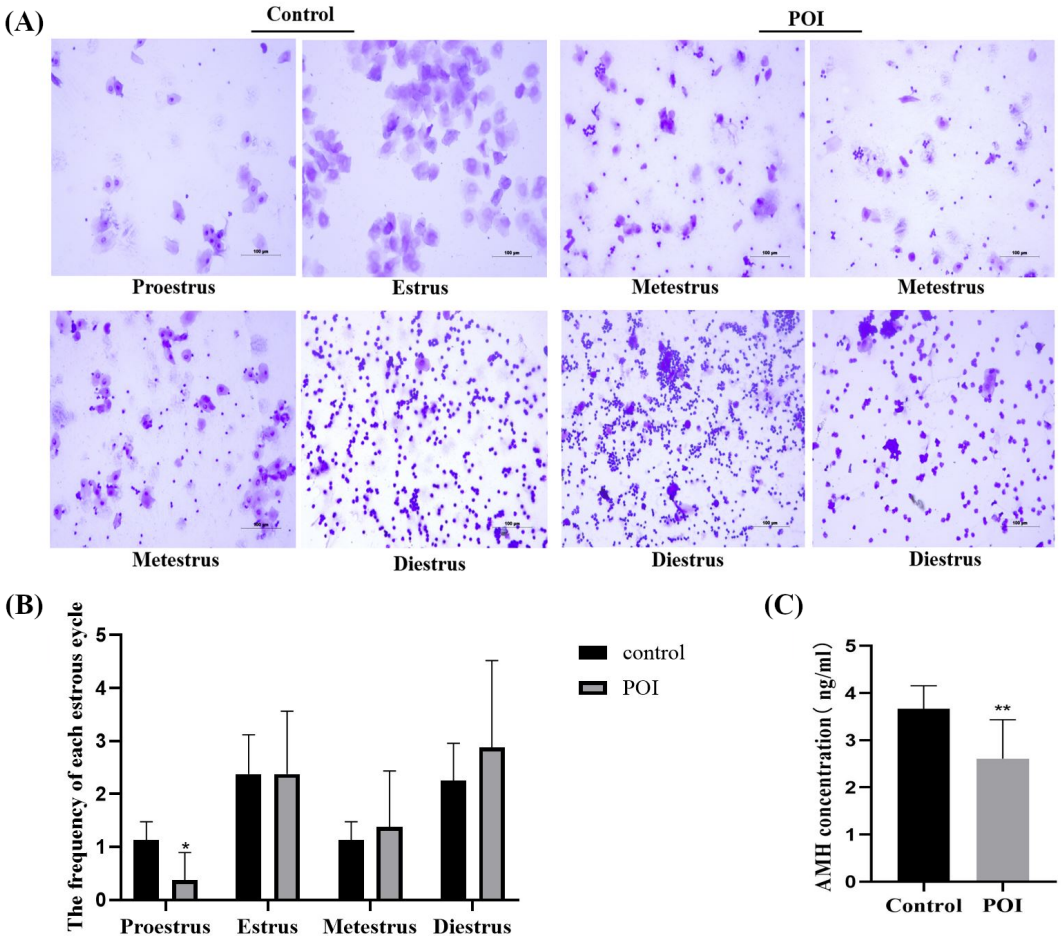


Figure 2. Assessment of estrous-cycle regularity and ovarian reserve function. (**A**) Representative photomicrographs of vaginal cytology at different estrous-cycle stages, including proestrus, estrus, metestrus, and diestrus, in Control and POI mice. Scale bars, 100 μm. (**B**) Statistical analysis of the frequency of each estrous cycle stage over the observation period. Data are presented as mean ± SD (n = 8 mice per group). (**C**) Serum anti-Müllerian hormone (AMH) concentrations in Control and POI mice. Data are presented as mean ± SEM (n = 8 mice per group). Statistical significance was determined using Student's t-test. *P < 0.05 and **P < 0.01 *vs*. Control group.

mice compared to controls (Figure 3). To investigate functional consequences, we modulated *HSD17B12* expression in KGN and SKOV3 cells. Fluorescence microscopy confirmed a transduction efficiency of approximately 80% (Supplementary Figure S1, *https://www.ddtjournal.com/action/getSupplementalData.php?ID=321*). Validation experiments demonstrated successful knockdown and overexpression of *HSD17B12* at both transcriptional and translational levels in stable cell lines (Figure 4). These results confirm that *HSD17B12* expression is significantly compromised in the POI context.

3.3. *HSD17B12* modulates cell proliferation and apoptosis

CCK-8 assays revealed that *HSD17B12* knockdown significantly inhibited proliferation in both KGN and SKOV3 cells at 24, 48, and 72 h, whereas overexpression promoted proliferation compared to respective controls (Figures 5A-B). Flow cytometry analysis indicated that *HSD17B12* knockdown markedly increased the apoptotic rate in KGN cells compared to NC (P = 0.006) and OE groups (P = 0.038); however, overexpression did not significantly reduce apoptosis below baseline levels (P = 0.179, Figure 5C). Similarly, in SKOV3 cells, knockdown induced significant apoptosis (P = 0.009 *vs*. NC; P = 0.034 *vs.* OE), while overexpression showed no significant protective effect against baseline apoptosis (P = 0.724, Figure 5D).

3.4. Transcriptomic profiling reveals immune and inflammatory dysregulation

RNA-seq analysis identified substantial transcriptional alterations upon *HSD17B12* modulation (Supplementary Figure S2, *https://www.ddtjournal.com/action/getSupplementalData.php?ID=321*). In KGN cells, comparison between OE and NC groups yielded 447 DEGs (289 upregulated, 158 downregulated; P < 0.001 , FC > 4.0), while sh *vs.* NC comparison revealed 1,171 DEGs (889 upregulated, 282 downregulated). In SKOV3 cells, 520 DEGs were identified in the OE group and 370 in the sh group (Figure 6).

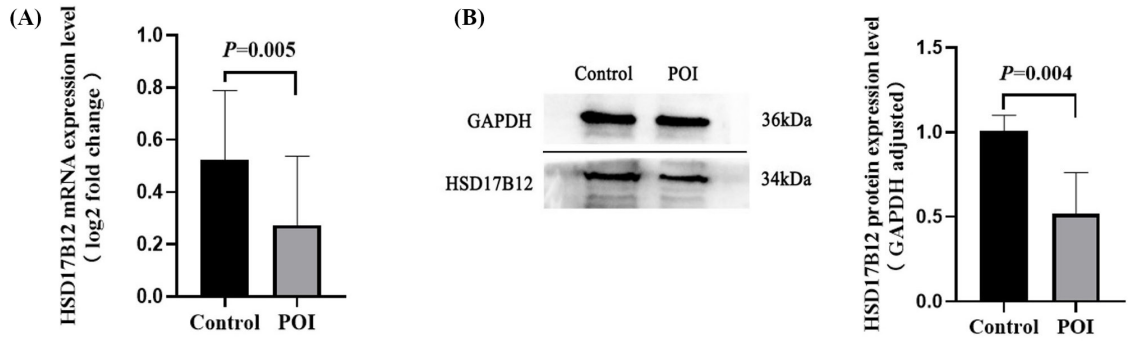


Figure 3. Expression of HSD17B12 in the ovaries of Control and POI mice. (**A**) Quantitative RT-PCR analysis of Hsd17b12 mRNA levels (n = 10 mice per group). (**B**) Representative Western blot image and densitometry quantification of HSD17B12 protein levels (n = 3 independent experiments). Data are presented as mean ± SD. Statistical significance was determined by Student's t-test. Specific P values are indicated in the graphs.

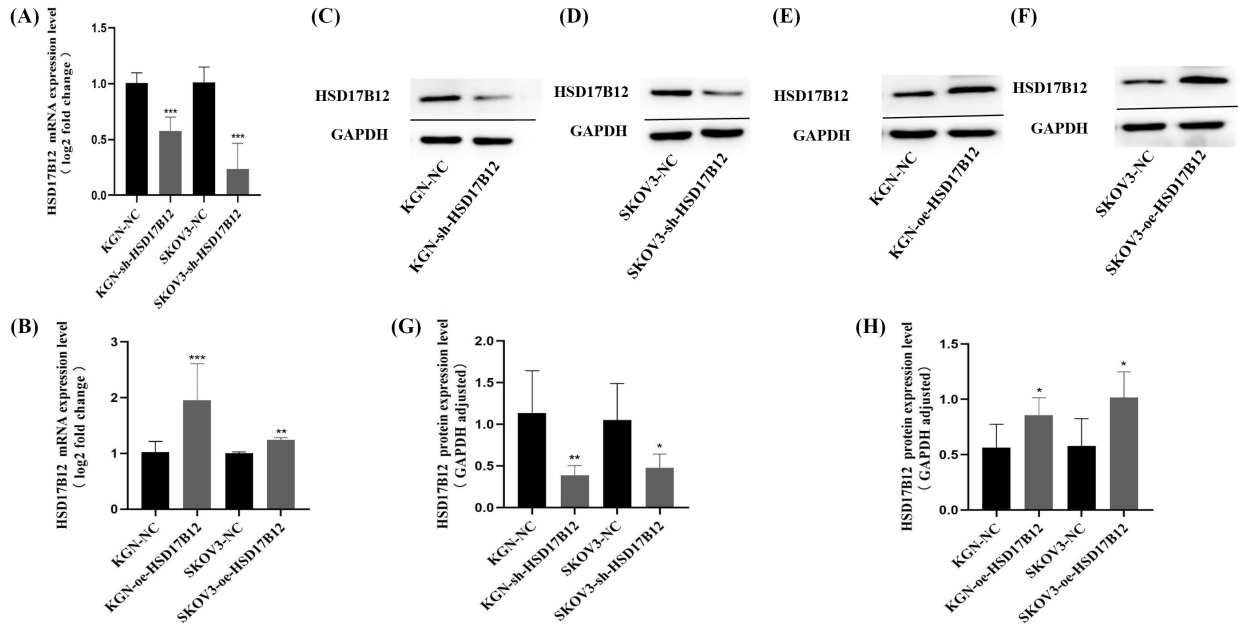


Figure 4. Validation of *HSD17B12* knockdown and overexpression in KGN and SKOV3 cells. (**A-B**) qRT-PCR analysis. (**C-H**) Representative Western blot images and densitometry quantification. Data are presented as mean ± SD from n = 3 independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. (*P < 0.05, **P < 0.01, ***P < 0.001).

To delineate the potential mechanism linking HSD17B12 to apoptosis, we focused on the immune and inflammatory pathways. KEGG analysis revealed significant enrichment in "Cytokine-cytokine receptor interaction' and 'NF-κB signaling pathway" (Figure 7). Furthermore, GO analysis highlighted a cluster of terms related to immune response, including MHC class II molecules, von Willebrand factor (vWF), and chemokine activity (Figure 8). Specifically, HSD17B12 knockdown led to the upregulation of key pro-inflammatory genes, suggesting that HSD17B12 deficiency may create a pro-inflammatory transcriptomic landscape that predisposes granulosa cells to apoptosis.

4. Discussion

POI pathogenesis is increasingly linked to aberrant granulosa cell dynamics, including impaired proliferation and excessive apoptosis, leading to follicular atresia. *HSD17B12* encodes a key enzyme in estrogen biosynthesis, converting estrone to the more potent estradiol, thereby regulating oogenesis and ovulation. Prior studies indicate that *Hsd17b12* haploinsufficiency in mice results in subfertility, smaller litter sizes, and meiotic arrest in immature follicles, likely mediated by disrupted arachidonic acid metabolism (*7*). Similarly, in Nile tilapia, *HSD17B12* is implicated in oocyte maturation *via* steroidogenesis (*9*). Consistent with these findings, our study demonstrates significant downregulation of *Hsd17b12* in a *Tripterygium wilfordii* polyglycoside suspension-induced POI mouse model. Functionally, *HSD17B12* overexpression promoted proliferation, whereas knockdown inhibited proliferation and increased apoptosis; however, overexpression did not

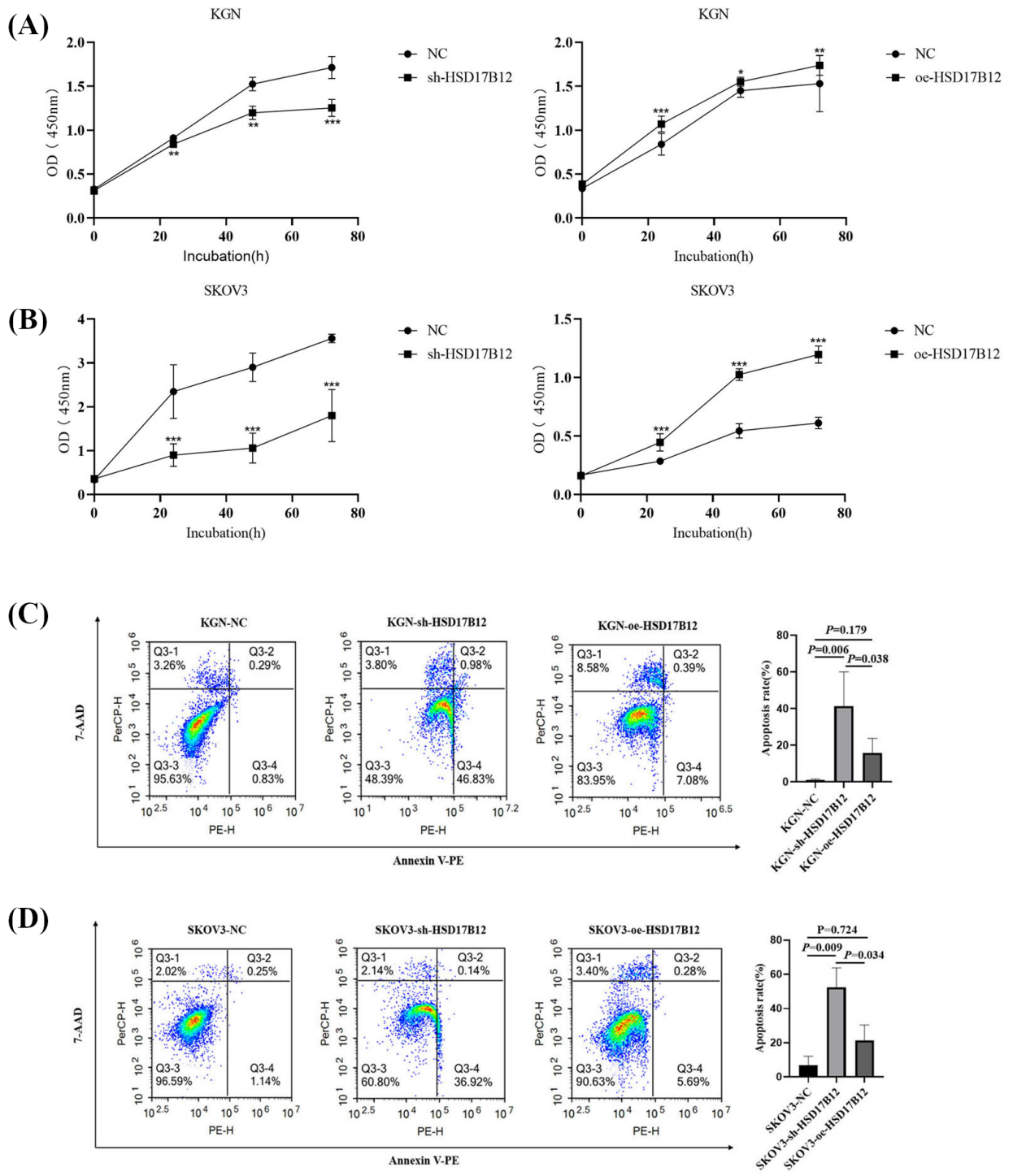


Figure 5. Effects of *HSD17B12* modulation on cell proliferation and apoptosis. (**A-B**) CCK-8 assays in KGN and SKOV3 cells at 0, 24, 48, and 72 h. The 0 h OD values were measured immediately after seeding and were comparable among groups. (**C-D**) Flow cytometric analysis of apoptosis. Data represent mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (one-way ANOVA with Tukey's test).

significantly reduce apoptosis under our experimental conditions.

Recent studies indicate that POI pathogenesis is closely associated with chronic inflammation. Elevated levels of inflammatory factors and cells in peripheral blood, ovarian tissue, and follicular fluid of POI patients suggest that GCs and oocytes are damaged *via* inflammatory mechanisms. M1 macrophages and their secreted pro-inflammatory cytokines can activate primary follicles, whereas M2 macrophages maintain follicular dormancy (*10*). Sun *et al.* (*11*) reported significantly elevated serum IL-6 and IL-21 levels in patients with primary POI compared with controls. Previous studies have confirmed elevated serum TNF-α, IL-1, and IL-6 levels in POI patients (*12*). Moreover, Acayak *et al.* (*13*) demonstrated that the neutrophil-

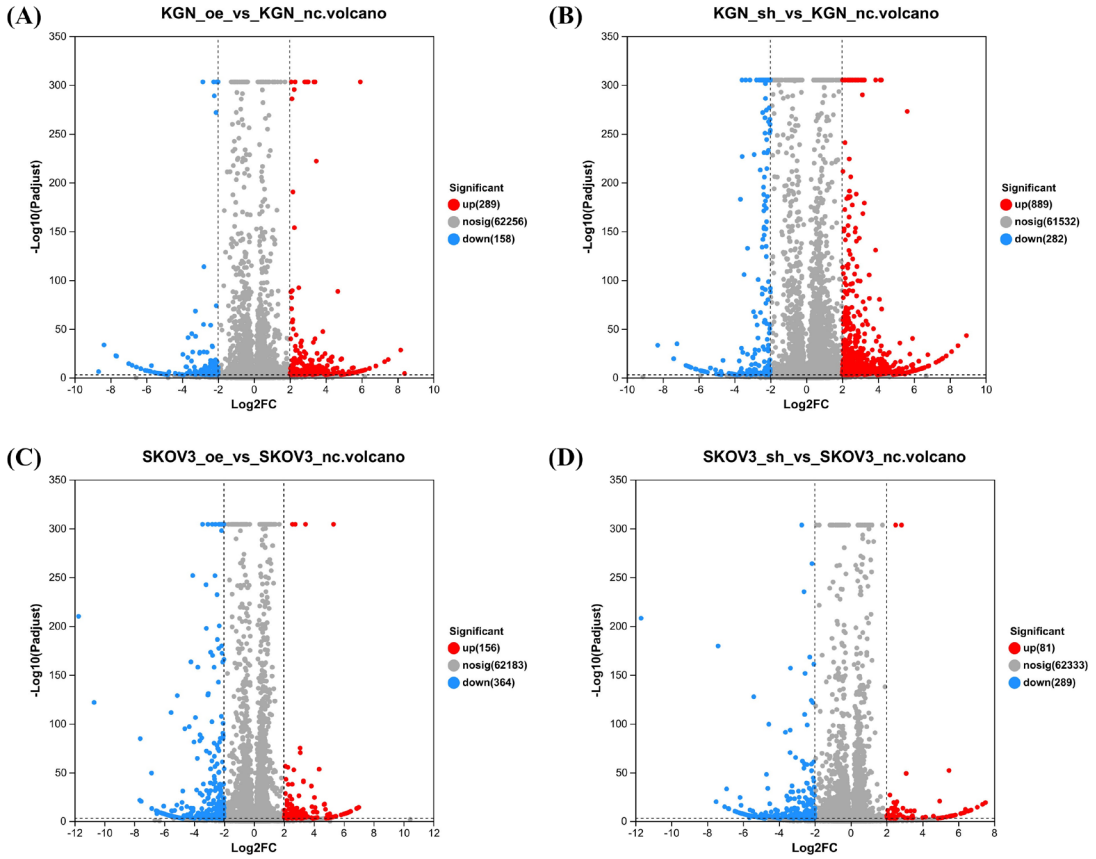


Figure 6. Volcano plots of differentially expressed genes (DEGs). Transcriptomic profiles of (**A**, **B**) KGN cells and (**C**, **D**) SKOV3 cells were analyzed. Comparisons were made between (**A**, **C**) Overexpression (OE) *vs.* Negative Control (NC) and (**B**, **D**) Knockdown (sh) *vs.* NC. Red dots represent significantly upregulated genes, blue dots represent significantly downregulated genes, and gray dots indicate non-significant genes. The screening criteria were set as $|\log_2 FC| \geqq 2$ and FDR < 0.05. The number of DEGs identified in each comparison is annotated in the legend (*e.g.*, 289 upregulated and 158 downregulated genes in KGN-OE *vs.* NC).

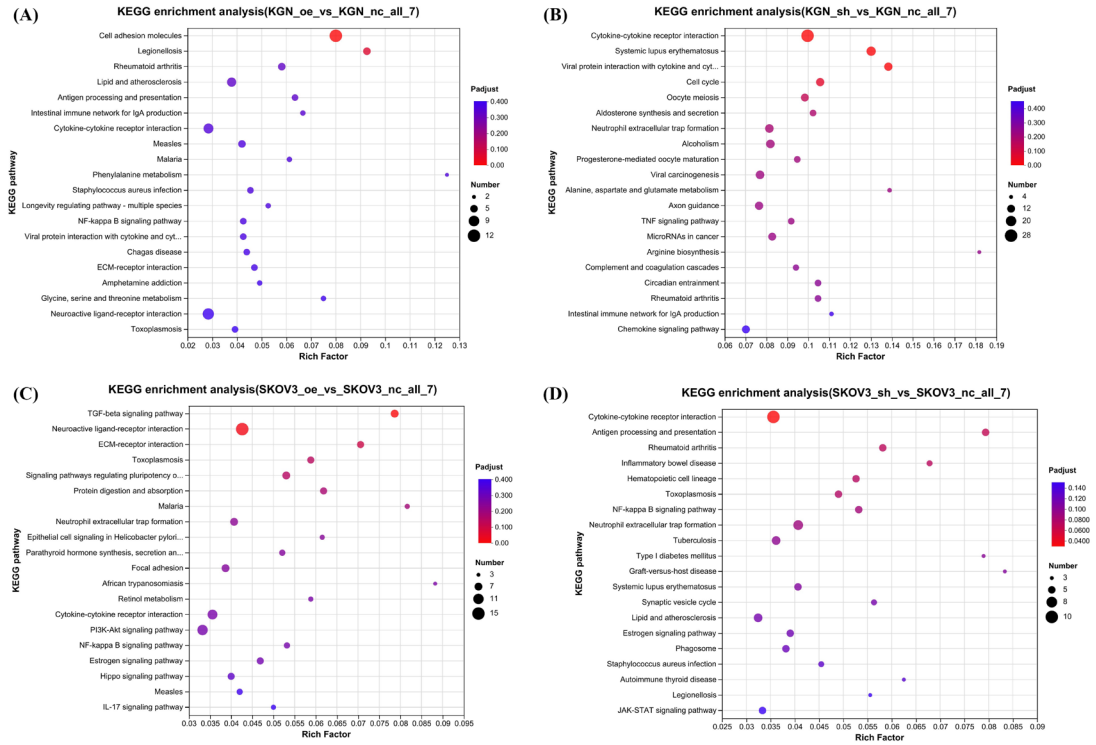


Figure 7. KEGG pathway enrichment analysis of DEGs. Top enriched pathways in KGN (**A, B**) and SKOV3 (**C, D**) cells comparing overexpression (OE) *vs.* negative control (NC) (**A, C**) and knockdown (sh) *vs.* NC (**B, D**). Dot size represents the number of enriched genes, and color intensity (from blue to red) indicates statistical significance (adjusted *P*-value).

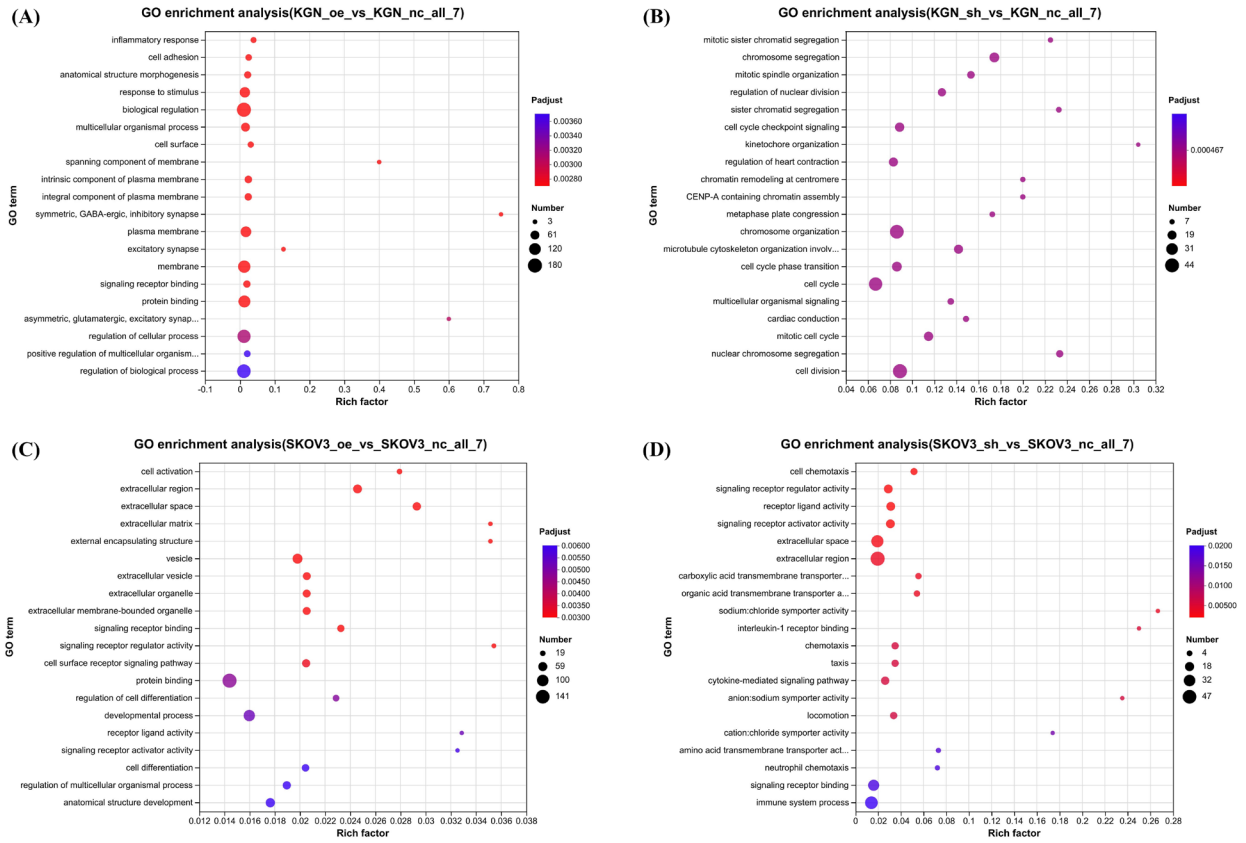


Figure 8. Gene Ontology (GO) biological process enrichment analysis. Top enriched GO terms in KGN (**A**, **B**) and SKOV3 (**C**, **D**) cells comparing overexpression (OE) *vs.* negative control (NC) (**A**, **C**) and knockdown (sh) *vs.* NC (**B**, **D**). Dot size represents the number of enriched genes, and color intensity (from blue to red) indicates statistical significance (adjusted *P*-value).

to-lymphocyte ratio (NLR), an indicator of systemic inflammation, was significantly higher in patients with idiopathic POI than in healthy controls, suggesting a link between inflammation and idiopathic POI. Within follicles, the elevated inflammatory status of GCs is significantly negatively correlated with oocyte quality (*14*). TNF-α in the follicle may upregulate the NF-κB pathway in GCs, increasing IL-1 and IL-6 secretion and thereby reducing oocyte quality. However, the detailed mechanisms by which these inflammatory components promote POI progression remain elusive.

Our transcriptomic data provides a novel perspective by identifying a strong correlation between *HSD17B12* deficiency and the activation of pro-inflammatory pathways. Specifically, in *HSD17B12* knockdown/overexpression cell models, RNA-seq analysis suggests that inflammatory and NF-κB-related pathways may participate in *HSD17B12* deficiency-induced cellular injury. While the precise molecular cascade requires further validation, we propose a novel mechanistic hypothesis (Figure 9): *HSD17B12* deficiency may act as an upstream metabolic trigger, leading to dysregulation of the ovarian immune microenvironment. Specifically, given HSD17B12's role in steroidogenesis and fatty acid metabolism (*15,16*), its downregulation could potentially alter metabolic intermediates that serve as ligands or

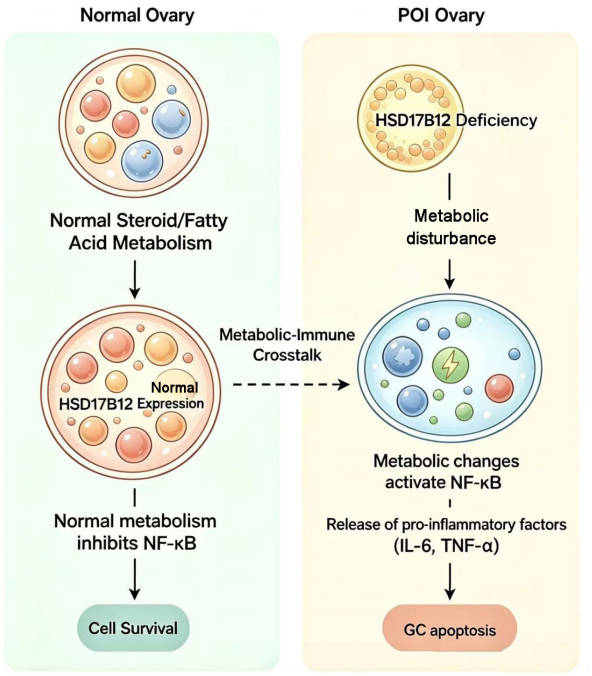


Figure 9. Schematic illustration of the proposed working model. *HSD17B12* deficiency may induce metabolic reprogramming and inflammatory transcriptomic changes, including NF-κB-related signatures, thereby contributing to granulosa cell apoptosis and POI development.

modulators for immune receptors, thereby activating the NF-κB pathway and ultimately inducing granulosa cell apoptosis (*17*).

Several limitations of this study should be acknowledged. First, regarding the model systems, while our chemical-induced mouse model demonstrated specific ovarian damage (evidenced by reduced AMH, disrupted estrous cycles, and follicular atresia), the observed weight loss suggests potential systemic toxicity, which may confound the interpretation of our results. Furthermore, although KGN cells are a well-established model for human granulosa cells, our *in vitro* findings using tumor-derived cell lines cannot fully recapitulate the complexity of primary human ovarian tissue. Second, in terms of research depth, this study represents a transcriptome-guided mechanistic hypothesis. We acknowledge that direct evidence, such as Western blot validation of p-NF-κB or rescue experiments using NF-κB inhibitors, is currently lacking. However, our identification of the "steroid metabolism-immune crosstalk" axis offers a new conceptual framework for understanding POI pathogenesis. Although *HSD17B12* modulation affected proliferation and apoptosis in both KGN and SKOV3 cells, KGN is the primary granulosa-like model, whereas SKOV3 is an ovarian cancer-derived epithelial context; therefore, the granulosa-cell relevance of our findings should be interpreted mainly on the basis of the KGN data. Despite these limitations, our study provides compelling evidence linking HSD17B12 deficiency to granulosa cell apoptosis and inflammatory dysregulation. Future studies involving patient samples and *in vivo* mechanistic interventions are necessary to fully delineate the therapeutic potential of targeting HSD17B12 in POI.

5. Conclusion

HSD17B12 expression is significantly altered in the ovaries of POI mice, directly affecting the proliferation and apoptosis of human ovarian cells. Furthermore, our findings suggest that HSD17B12 contributes to POI pathogenesis by modulating immune and inflammatory response pathways, highlighting a potential "steroid metabolism-immune crosstalk" axis. Future studies involving patient samples and *in vivo* mechanistic interventions are necessary to fully delineate the therapeutic potential of targeting HSD17B12 in POI.

Funding: This work was supported by a grant from the Inner Mongolia Grassland Talents Innovation Team Project (2022), Translational medicine project of Inner Mongolia Medical University, Inner Mongolia Medical University doctoral program construction unit fund matching project (RC2300002962), Inner Mongolia Autonomous Region Science and Technology Plan Project, granted by Inner Mongolia Reproductive Health Research Center (2022YFSH0067,2023KJHZ0038), Inner Mongolia Academy of Medical Sciences public hospital research joint fund project (2024GLLH0368) and Shandong Province Medical and Health Science and Technology Project (202405010322).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received March 3, 2026; Revised June 18, 2026; Re-revised September 3, 2026; Accepted September 20, 2026.

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Released online in J-STAGE as advance publication October 29, 2026.

Brief Report

DOI: 10.5582/ddt.2026.01061

Hot-water extraction, ethanol precipitation, and silkworm bioassays detect innate immune-stimulating activity in coffee extracts

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SUMMARY: A workflow combining hot-water extraction and ethanol precipitation can provide macromolecule-enriched fractions, but rapid *in vivo* evaluation of their innate immune-stimulating activity is not well established. We used coffee as a test matrix and evaluated the resulting fractions using a silkworm muscle-contraction assay and a *Pseudomonas aeruginosa* infection assay. Ethanol-precipitated fractions were prepared from three coffee origins, with or without lactic acid bacterial fermentation, under light- or dark-roast conditions. All sample groups showed measurable muscle-contraction activity. Specific activity ranged from 0.74 to 9.1 U/mg and was higher after light roasting in 11 of 12 matched conditions. Selected fractions increased survival at approximately 24 h after *P. aeruginosa* challenge, most clearly among Colombia EX samples; protection varied with bean origin, fermentation condition, roast, and dose. Non-fermented fractions were also active, indicating that fermentation was not uniformly required. These results support the feasibility of combining hot-water extraction, ethanol precipitation, and silkworm testing to detect bioactivity in macromolecule-enriched fractions. The method may be useful for prioritizing samples for chemical characterization.

Keywords: *Bombyx mori*, *Pseudomonas aeruginosa*, prophylaxis, polysaccharide

1. Introduction

Hot-water extracts can contain diverse water-soluble macromolecules, but practical workflows for enriching these materials and evaluating their *in vivo* biological activity remain limited. Coffee was selected as a test matrix because brewed coffee contains substantial soluble dietary fiber, including galactomannan- and arabinogalactan-related structures, whose abundance and fine structure depend on bean type, roasting, and brewing conditions (*1-3*). Ethanol precipitation has also been used to recover a coffee polysaccharide fraction that stimulated macrophage and intestinal immune readouts (*4*). These findings support the use of coffee to evaluate a workflow for biological screening of macromolecule-enriched fractions.

Silkworm bioassays can provide an intact-host component of such a screening workflow. In the silkworm *Bombyx mori*, the cytokine paralytic peptide is produced as an inactive precursor and is activated after tissue damage or recognition of microbial cell-wall components (*5-8*). Activated paralytic peptide induces cellular and humoral immune responses, and polysaccharide-dependent activation can be quantified using the silkworm muscle-contraction assay (*7-10*).

Silkworm infection models also provide functional readouts of host protection and have been used to study immune priming, lactic acid bacteria, bacterial virulence, and anti-infective agents (*11-20*). In particular, Miyashita *et al.* demonstrated that Gram-negative peptidoglycan or ingested bacterial stimuli can induce resistance to subsequent *P. aeruginosa* infection (*11,12*). Because lactic acid bacteria have been reported to activate innate immunity in silkworms (*13*), fermented and non-fermented coffee beans were included to examine whether fermentation was associated with the activity of the recovered fractions. We therefore examined whether a workflow combining hot-water extraction, ethanol precipitation, and silkworm testing could detect innate immune-stimulating activity, using coffee as a proof-of-concept matrix.

2. Materials and Methods

2.1. Coffee preparation, fermentation, and hot-water extraction

Green coffee beans from Guatemala SHB, Vietnam G1,

and Colombia EX were examined. Where indicated, beans were incubated with *Lactococcus lactis* 11/19-B1, *Weissella hellenica* 0916-4-2, or *Enterococcus faecalis* YM0831. Non-inoculated beans were included as comparators. Beans were roasted under light- and dark-roast conditions, ground, and extracted with hot water. Bean origin, fermentation condition, and roast were treated as sample descriptors rather than independent causal factors.

2.2. Ethanol precipitation

Two volumes (100 mL) of absolute ethanol were added to 50 mL of coffee extract. After incubation on ice for 1 h, precipitates were recovered by centrifugation (8,000 rpm, 10 min), washed with ethanol, dried, and redissolved in 5 mL of physiological saline. This operational fraction was expected to enrich macromolecules but was not chemically homogeneous.

2.3. Silkworm rearing

Silkworm eggs (*Bombyx mori*, strain KINSYU × SHOWA) were obtained from Ehime-Sanshu (Ehime, Japan). The larvae were reared at 27°C on an artificial diet (Silkmate 2S). On the first day of the fifth instar, each larva was provided with approximately 1 g of the diet, and larvae on the second day of the fifth instar were used for the experiments.

2.4. Silkworm muscle-contraction assay

Two-fold serial dilutions of each fraction were injected into isolated silkworm muscle specimens according to the established assay (*9*). Contraction was calculated as C = (initial length − post-injection length)/initial length. One unit was defined as the dose producing C = 0.15, and specific activity was expressed as U/mg of recovered material.

2.5. Silkworm *P. aeruginosa* infection assay

The silkworm infection assay was performed based on the methods described by Miyashita *et al.* (*11,12*). Silkworm larvae received ethanol-precipitated fractions at 0.5 or 5.0 mg/mL before being challenged with 50 µL of a 10^6-fold dilution of an overnight culture of *P. aeruginosa* PAO1. Physiological saline alone was administered before PAO1 challenge as the vehicle-treated, infected control. Survival was recorded approximately 24 h after infection. Each condition contained four larvae (n = 4). Survival results are presented descriptively as percentages without inferential statistical analysis.

3. Results and Discussion

3.1. Muscle-contraction activity

All sample groups yielded measurable activity, with specific activities ranging from 0.74 to 9.1 U/mg (Figure 1). Light-roast fractions were more active than their corresponding dark-roast fractions in 11 of 12 matched conditions; the exception was Vietnam G1 fermented with *E. faecalis* YM0831. The highest activity was observed for light-roasted Guatemala SHB fermented

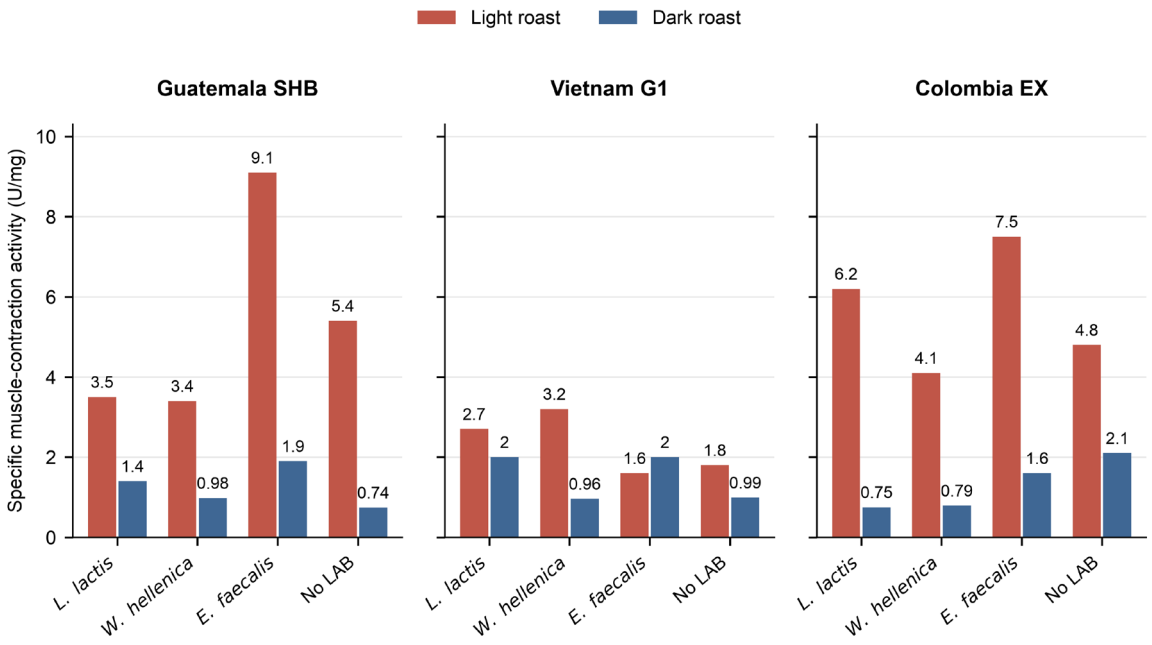


Figure 1. Specific silkworm muscle-contraction activity of ethanol-precipitated coffee fractions. Panels show Guatemala SHB, Vietnam G1, and Colombia EX fractions under the indicated fermentation and roast conditions. Numerical values above bars are specific activities in U/mg of recovered material. No LAB indicates the non-fermented comparator.

with *E. faecalis* YM0831 (9.1 U/mg), followed by light-roasted Colombia EX fermented with *E. faecalis* YM0831 (7.5 U/mg) and *L. lactis* 11/19-B1 (6.2 U/mg). Non-fermented controls were also active. Thus, ethanol-precipitable coffee constituents can stimulate the paralytic peptide-linked assay, consistent with previous work on immunostimulatory polysaccharides and silkworm cytokine activation (*6-10*), but fermentation was not uniformly required.

3.2. Prophylactic activity against *P. aeruginosa*

Survival in the saline-pretreated, PAO1-infected control group was 0/4 (0%). In the silkworm *P. aeruginosa* infection assay, survival at 0.5 mg/mL was limited for Guatemala SHB and Vietnam G1 fractions, whereas several Colombia EX fractions produced 50-100% survival (Figure 2). At 5.0 mg/mL, protective activity was broader: most Vietnam G1 and Colombia EX conditions produced 50-100% survival, while Guatemala SHB remained comparatively weak. Both fermented and non-fermented preparations were active in some conditions. These results do not establish fermentation as the sole determinant of survival. Muscle-contraction activity did not consistently predict survival. For example, light-roasted Guatemala SHB fermented with *E. faecalis* YM0831 had the highest specific activity (9.1 U/mg), but survival was 0% at both tested concentrations. The two assays measure different outcomes, and activity in the muscle-contraction assay does not necessarily translate into protection against infection.

3.3. Utility and limitations of the workflow

The principal outcome is methodological. When applied to coffee as a test matrix, the combined workflow produced ethanol-precipitated fractions that retained activity in two complementary silkworm assays. The muscle-contraction assay supplied a rapid quantitative readout, whereas the infection assay measured survival after bacterial challenge. Silkworm models are inexpensive, accommodate direct administration, and have supported infection biology and drug-discovery studies across bacterial and fungal pathogens (*14-20*). These features make the paired assays potentially useful for prioritizing ethanol-precipitated fractions from hot-water extracts before detailed purification.

The study has limitations. The muscle-contraction results were evaluated descriptively without inferential statistical analysis. The infection experiment used four larvae per group. Direct inhibition of *P. aeruginosa* by the coffee fractions was not excluded; improved survival therefore cannot be attributed specifically to host immune stimulation. Endotoxin and microbial carryover

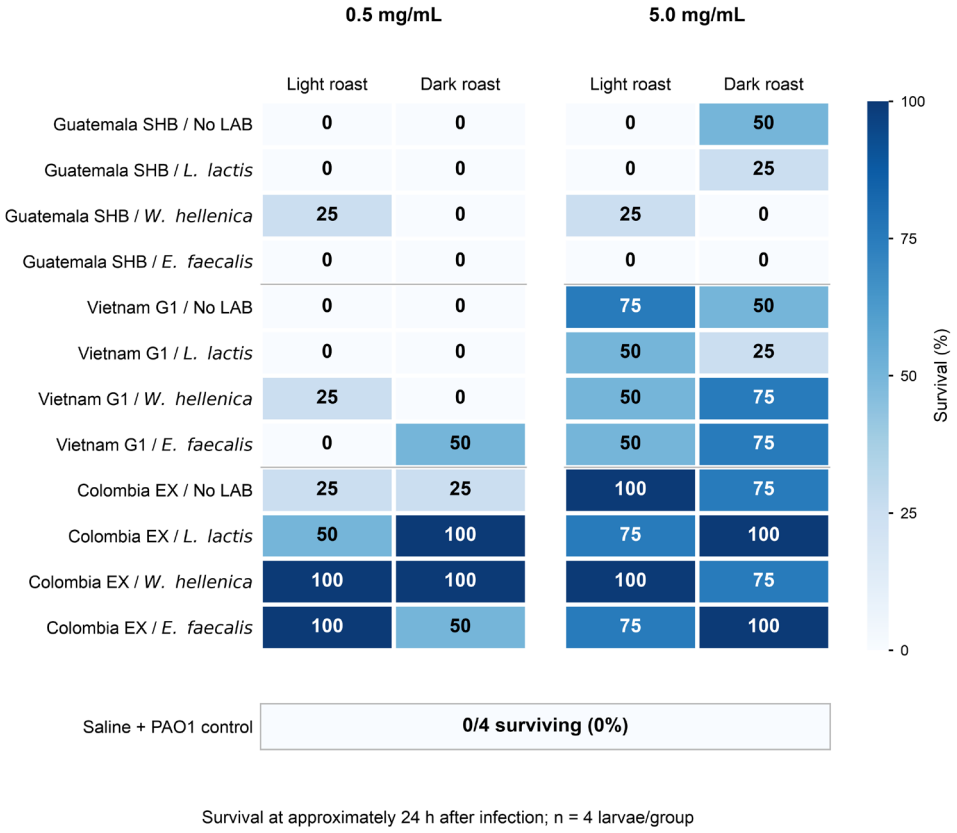


Figure 2. Prophylactic activity of ethanol-precipitated coffee fractions in the silkworm *Pseudomonas aeruginosa* infection model. Heatmaps show survival percentages at approximately 24 h after infection following pretreatment with 0.5 or 5.0 mg/mL fractions. Each cell represents four larvae. The saline-pretreated, PAO1-infected control is shown separately and had 0/4 surviving larvae (0%). No LAB indicates the non-fermented comparator.

were not quantified, and ethanol precipitation may recover polysaccharides, glycoproteins, aggregates, and other complexes. Generalization to tea, broth, or other beverages requires direct testing. Future studies should include individual-level data, measure extraction yield, include endotoxin controls, and chemically fractionate only samples with reproducible activity.

In conclusion, a workflow combining hot-water extraction, ethanol precipitation, and silkworm bioassays detected muscle-contraction activity and increased survival after bacterial challenge in selected groups treated with ethanol-precipitated fractions prepared from a hot-water extract. Coffee served as a proof-of-concept matrix; application to other hot-water extracts requires direct evaluation.

Acknowledgements

This study was supported by Genome Pharmaceuticals Institute Co., Ltd.

Funding: This work was supported by METI R&D Support Program for Growth-oriented Technology SMEs Grant Number JPJ005698 to Kazuhisa Sekimizu.

Conflict of Interest: Kazuhisa Sekimizu was an advisor to Genome Pharmaceuticals Institute Co., Ltd. The other authors declare no competing interests.

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Received August 26, 2026; Revised October 8, 2026; Accepted October 12, 2026.

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Released online in J-STAGE as advance publication October 29, 2026.

Brief Report

DOI: 10.5582/ddt.2026.01062

Collagen-derived peptides enhance resistance to *Pseudomonas aeruginosa* infection in silkworms

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SUMMARY: Collagen-derived peptides are widely used as food ingredients and dietary supplements, but their effects on host defense against bacterial infection remain poorly understood. Here, we examined whether GFG-01, a granulated preparation of a fish-derived collagen hydrolysate produced using ginger protease, enhances resistance to *Pseudomonas aeruginosa* PAO1 infection in the silkworm (*Bombyx mori*). Oral administration of GFG-01 by mixing the preparation into the diet improved the post-infection outcome of larvae compared with vehicle-treated controls. Western blot analysis suggested that intrahemocoelic administration of GFG-01 induced accumulation of Cecropin B, an antimicrobial peptide, in the hemolymph. RNA-seq-based Gene Ontology enrichment analysis of samples collected after oral administration further showed tissue-dependent immune-related gene expression patterns: immune-related categories were enriched among downregulated genes in the fat body and among upregulated genes in hemocytes. These findings indicate that orally administered GFG-01 can improve the outcome of bacterial infection in silkworms and that GFG-01 is associated with modulation of host innate immune responses under the tested conditions.

Keywords: *Bombyx mori*, GFG-01, Cecropin B, innate immunity, RNA sequencing, functional food

1. Introduction

Collagen-derived peptides are widely used as food ingredients and dietary supplements, but their effects on host defense remain poorly defined. More broadly, food-derived molecules are increasingly recognized not only as nutrients but also as modulators of host physiology and immunity. Indeed, several food- or microbiota-derived substances have been reported to stimulate innate immune responses in invertebrates and mammals, including high-molecular-weight broccoli-derived polysaccharides, peanut triacylglycerols, and human gut-associated bacteria identified by silkworm-based screening approaches (*1-4*). However, whether collagen-derived peptides can alter infection resistance at the whole-organism level remains unclear.

Appropriate *in vivo* models are required to evaluate such host-directed effects. We and others have established the silkworm (*Bombyx mori*) as an alternative infection model for studying host-pathogen interactions and therapeutic responses *in vivo* (*5-10*). In silkworms, injection of human pathogenic microorganisms into the hemolymph causes lethal infection, and survival can be used as a quantitative readout for infection progression and therapeutic efficacy (*5,6*). This model is inexpensive, experimentally tractable, and applicable to a wide range of pathogens, including bacteria and fungi (*7-10*).

Importantly, the silkworm infection model can be used not only to detect direct antimicrobial effects but also to analyze infection resistance mediated by activation of host innate immunity. Oral administration of lactic acid bacteria has been reported to improve survival in bacterial infection models of silkworms (*11*). In addition, Gram-negative bacterial peptidoglycan and ingested bacteria can induce primed immune responses that increase resistance to subsequent systemic infection (*12,13*). More recently, pretreatment with *N*-acetylchitohexaose was shown to enhance resistance to *P. aeruginosa* infection and to induce immune effector genes at the hemocyte transcriptome level (*14*). These phenomena are commonly discussed in the context of immune priming in insects (*15*).

A key consideration in interpreting such responses is that insect immunity is organized across multiple tissues. In silkworms and other insects, the fat body is a major source of humoral immune effectors, the gut serves as a frontline barrier for orally encountered materials, and hemocytes mediate cellular defenses such as phagocytosis, nodulation, and encapsulation (*16,17*). Studies in *Drosophila* further suggest that gut-derived signals can influence systemic immunity through inter-organ communication (*18,19*). These observations raise the possibility that orally administered immunomodulators may not induce a uniform whole-body immune response, but instead provoke distinct responses in different immune-related tissues.

This possibility is supported by recent tissue-resolved studies. Comparative transcriptomic analysis in *Drosophila* has shown that hemocytes and fat body, despite being the two principal professional immune tissues, can display strikingly different responses to systemic infection (*20*). In addition, the insect fat body is a polyfunctional tissue that integrates immunity with metabolism, storage, and reproductive physiology, and constraints on this multifunctionality can give rise to reproduction-immunity trade-offs (*21*). Therefore, changes in immune gene expression in one tissue do not necessarily predict organism-level protection, and improved infection resistance may arise from differently directed responses across tissues rather than from uniform immune activation.

Collagen-derived peptides are plausible candidates for such orally active host modulators precisely because their reported biological behavior is compatible with non-uniform, tissue-dependent immunoregulation. After oral ingestion of collagen hydrolysates, multiple hydroxyproline-containing oligopeptides are detected in human blood, indicating that orally administered collagen peptides are systemically bioavailable in peptide form (*22*). Moreover, some collagen-derived peptides can reach peripheral tissues after ingestion (*23*). Importantly, the reported immunological effects of collagen-derived peptides are context- and cell type-dependent rather than uniformly stimulatory (*24,25*).

Among insect innate immune effectors, antimicrobial peptides are major defense factors. Cecropins are evolutionarily conserved antimicrobial peptides induced by microbial challenge, and cecropin genes have been characterized in silkworms (*26,27*). Induction of such peptides is therefore an important indicator when evaluating whether collagen-derived peptides modify infection resistance through host responses.

Based on these considerations, we investigated whether orally administered GFG-01, a granulated preparation of a fish-derived collagen hydrolysate produced using ginger protease, improves the outcome of *P. aeruginosa* infection in silkworms. In separate experiments, we examined whether intrahemocoelic GFG-01 administration induced Cecropin B accumulation in the hemolymph and whether oral administration produced tissue-dependent immune-related gene expression.

2. Materials and Methods

2.1. Silkworms

Silkworm (*Bombyx mori*) larvae were reared as described previously (*5,6*). Unless otherwise stated, fifth-instar larvae were used. After sample pretreatment, larvae were maintained at 27°C before infection challenge and at 37°C after infection challenge.

2.2. Bacterial strain

Pseudomonas aeruginosa PAO1 was used as the infectious strain. A full-growth culture was diluted 10^{-6} in saline, and 50 μL was injected into the hemolymph as described for the silkworm infection model (*5,6*). The viable count of the undiluted culture was 2×10^9 CFU/mL, corresponding to an inoculum of 100 CFU/larva after dilution and injection.

2.3. GFG-01 preparation

GFG-01 was supplied by Nippi, Inc. (Tokyo, Japan). According to the manufacturer, GFG-01 is a granulated preparation of GFF-01, a fish-derived collagen hydrolysate prepared using ginger protease. GFF-01 and the preparation of ginger-protease-degraded collagen hydrolysates have been described previously (*28,29*). GFG-01 was mixed into the diet for oral administration or dissolved in saline for intrahemocoelic administration.

2.4. Oral administration and infection challenge

GFG-01 (1.018 g) was mixed with 6 g of artificial diet and administered orally to a group of five day-1 fifth-instar larvae at 27°C for 15 h. This amount was selected based on preliminary dose-ranging experiments. The amount consumed was estimated from the difference between the initial and remaining diet weights, corresponding to an estimated intake of 99 mg GFG-01/larva. Vehicle-treated controls received diet prepared under the same conditions without GFG-01. After feeding, each larva was challenged intrahemocoelically with 50 μL of a 10^{-6} dilution of a full-growth *P. aeruginosa* PAO1 culture. Larvae were maintained at 37°C, and survival was assessed 24 h after infection.

2.5. Western blotting

GFG-01 (500 μg/larva in 50 μL saline) or 50 μL saline was injected into the hemolymph of fifth-instar larvae. After incubation at 27°C for 6 h, hemolymph was collected from six larvae in each group and pooled in

pairs to generate three samples per group. Proteins were precipitated, separated by SDS-PAGE, and transferred to PVDF membranes. Cecropin B was detected using a rabbit anti-Cecropin B antibody (Abcam, ab27571; 1:1,000) and horseradish peroxidase-conjugated goat anti-rabbit IgG (SouthernBiotech, 4030-05; 1:4,000), followed by chemiluminescent detection.

2.6. RNA-seq and GO enrichment

Fat body and hemocyte samples were collected from fifth-instar larvae 15 h after oral administration of GFG-01 at an estimated intake of 76 mg/larva or control diet. One larva was used for each biological replicate, and three biological replicates were analyzed per condition for each tissue. Fat bodies were dissected, collected with forceps, and homogenized before RNA extraction. Hemolymph was collected into tubes containing phenylthiourea to prevent melanization, and hemocytes were recovered by centrifugation. Total RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research, R2050), and RNA concentration was measured using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Q32852). Library preparation and mRNA sequencing on an Illumina NovaSeq X Plus platform, generating approximately 6 Gb per sample, were performed as described by Mikami *et al.* (*14*), with modifications. Transcript abundance was quantified using Salmon version 1.10.3 and imported with tximport. Genes with counts of at least 10 in three or more samples were retained for analysis. Differential expression analysis was performed using DESeq2 version 1.43.1 in R version 4.5.0. Differentially expressed genes were defined by an adjusted P value < 0.05 and an absolute $\log_2$ fold change ≥ 1. Genes were annotated using SilkDB3.0 annotation files. GO enrichment analysis was performed using the enricher function of clusterProfiler version 4.16.0 with Benjamini-Hochberg correction. The feeding conditions were the same in the infection and RNA-seq experiments; the different estimated intakes reflect differences in actual diet consumption.

2.7. Statistical analysis

Survival at the 24-h endpoint was compared using a two-sided Fisher's exact test. For RNA-seq, differential expression and enrichment significance were evaluated as described above. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Oral administration of GFG-01 improves post-infection larval condition

We first asked whether GFG-01 could affect the course of bacterial infection when administered through a physiologically relevant oral route. GFG-01 was mixed into the diet and administered for 15 h before intrahemocoelic challenge with *P. aeruginosa* PAO1. This design allowed us to test whether prior oral exposure to GFG-01 could alter the subsequent course of systemic bacterial infection. At the estimated intake of 99 mg/larva shown in Figure 1, all vehicle-treated infected larvae died within 24 h (0/5 survived), whereas all GFG-01-treated larvae survived and remained visibly healthier (5/5; two-sided Fisher's exact test, P = 0.0079). These observations indicate that oral administration of GFG-01 can improve the outcome of *P. aeruginosa* infection in silkworms.

3.2. Intrahemocoelic GFG-01 administration induces Cecropin B accumulation in the hemolymph

We next examined whether GFG-01 could induce an antimicrobial peptide response independently of gastrointestinal processing. GFG-01 was administered intrahemocoelically, and hemolymph was collected 6 h later for western blot analysis using an antibody against Cecropin B. This assay was used because Cecropin B is a representative antimicrobial peptide involved in insect humoral innate immunity. Western blot analysis showed a Cecropin B-positive band in GFG-01-treated larvae, whereas saline-treated controls showed a weak or absent corresponding signal under the same processing conditions (Figure 2). These results suggest that intrahemocoelic administration of GFG-01 induces the accumulation of Cecropin B, an antimicrobial peptide, in the hemolymph.

3.3. GFG-01 treatment is associated with tissue-dependent immune-related transcriptional patterns

Because insect immune responses are distributed across multiple tissues, we then asked whether orally administered GFG-01 produced similar or distinct transcriptional responses in major immune-related tissues. Fat body and hemocyte samples were collected 15 h after administration of GFG-01 at an estimated intake of 76 mg/larva or control diet and analyzed by RNA-seq. Differentially expressed genes were then subjected to Gene Ontology (GO) enrichment analysis. GO enrichment analysis revealed different immune-related expression patterns between the fat body and hemocytes (Figure 3). In the fat body, downregulated genes were enriched for terms including innate immune response and defense response to bacterium. In contrast, upregulated genes in hemocytes were enriched for similar immune-related categories. Thus, at 15 h after oral administration, the transcriptomic response to GFG-01 was not uniform across tissues.

4. Discussion

These findings support three main conclusions.

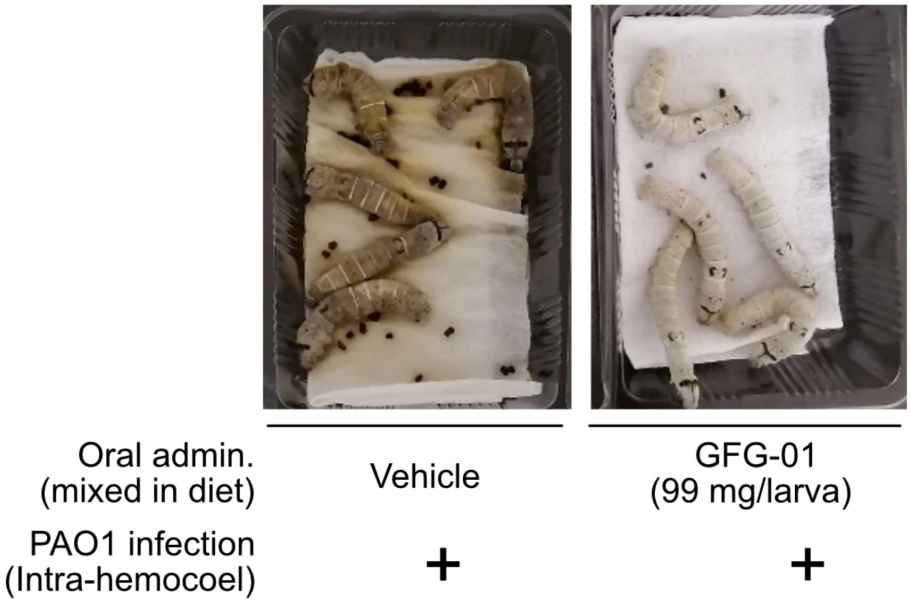


Figure 1. Oral administration of GFG-01 improves post-infection larval condition. GFG-01 was mixed into the diet and administered orally for 15 h at 27°C before intrahemocoelic infection with *P. aeruginosa* PAO1. Five larvae were examined per group. The estimated GFG-01 intake was 99 mg/larva. At 24 h after infection, none of the vehicle-treated larvae survived (0/5), whereas all GFG-01-treated larvae survived and remained visibly healthier (5/5; two-sided Fisher's exact test, $P = 0.0079$).

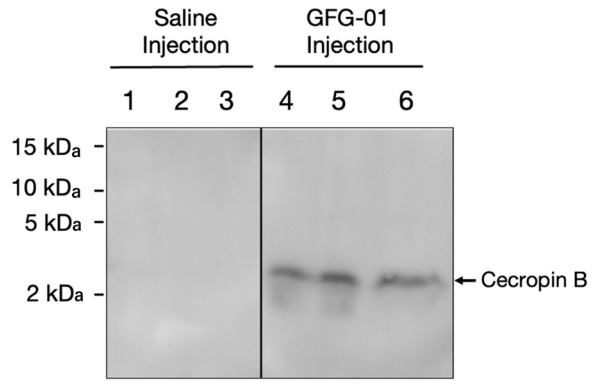


Figure 2. Intrahemocoelic GFG-01 administration induces Cecropin B accumulation in the hemolymph. Western blot of hemolymph collected from six larvae in each group 6 h after intrahemocoelic administration of saline or GFG-01 (500 µg/larva). Hemolymph from two larvae was pooled for each lane. Lanes 1-3, saline-treated samples; lanes 4-6, GFG-01-treated samples. A band in the expected Cecropin B region is observed in the GFG-01-treated lanes.

First, oral administration of GFG-01 improves the outcome of *P. aeruginosa* infection in silkworms under defined conditions. Second, intrahemocoelic GFG-01 administration induces Cecropin B accumulation in the hemolymph. Third, oral GFG-01 administration produces immune-related transcriptional responses that differ between the fat body and hemocytes rather than showing a uniform systemic pattern.

The transcriptomic data further support the view that the protective state is not explained by a single uniform immune program. At 15 h after oral administration, the fat body and hemocytes showed opposite trends in immune-related gene categories. Because these tissues play different roles in insect immunity, such non-parallel behavior is biologically plausible (*16-21*). Additional time-course analysis will be useful to determine whether these tissue responses represent different regulatory directions or different phases of a shared dynamic process.

The fat body findings require particularly cautious interpretation. Reduced expression of immune-related gene sets in the fat body might appear inconsistent with improved infection outcome. However, the fat body is a multifunctional tissue integrating immunity with metabolic and physiological functions (*16,21*). Reduced immune-related transcription at a single time point may reflect temporal resolution of earlier responses, tissue resource balancing, or selective pathway usage.

The accumulation of Cecropin B in the hemolymph after intrahemocoelic GFG-01 administration is consistent with previous studies linking cecropin induction to immune priming and enhanced infection resistance in silkworms (*12,13*). When considered together with the RNA-seq results, these findings raise the possibility that GFG-01 does not simply increase overall immune activity but instead alters the balance of immune responses among tissues. Increased defense-related transcription in hemocytes could contribute to infection resistance even when immune-related transcription in the fat body is reduced. However, Cecropin B was examined after intrahemocoelic administration, whereas RNA-seq was performed after oral administration; therefore, these findings cannot yet be placed within a single mechanistic sequence. Whether oral GFG-01 also induces Cecropin B remains to be

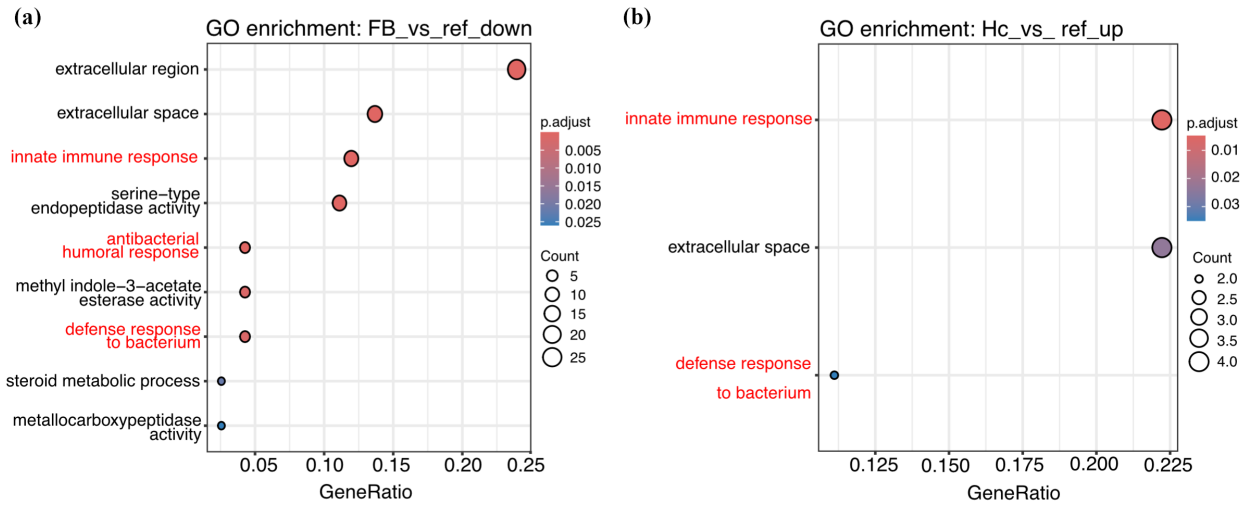


Figure 3. Tissue-dependent GO enrichment patterns are observed after oral GFG-01 administration. Fat body and hemocytes were collected 15 h after oral GFG-01 (76 mg/larva) or control diet (n = 3 larvae per condition and tissue; one larva per replicate). (**a**) GO enrichment of downregulated fat-body genes (FB_vs_ref_down). (**b**) GO enrichment of upregulated hemocyte genes (Hc_vs_ref_up). Immune-related terms are highlighted.

determined.

Further work will be needed to identify the active molecular species in GFG-01 and clarify the mechanisms underlying the enhanced infection resistance observed after oral administration. Nevertheless, the present results demonstrate that GFG-01 can improve the outcome of bacterial infection in an invertebrate host and support the use of silkworms as a tractable model for analyzing food-derived modulators of host defense *in vivo*.

Acknowledgements

We thank Genome Pharmaceuticals Institute Co., Ltd. for technical support.

Funding: This work was supported by JSPS KAKENHI (Grant #21H02733 and #24K02155) to K.S. This work was also supported by Nippi, Inc. and by the Open Innovation Research and Application Promotion Program (JPJ011937, Research Project No. 05001a1a2) from the Bio-oriented Technology Research Advancement Institution (BRAIN) (to A.M.).

Conflict of Interest: Kazuhisa Sekimizu was an advisor to Genome Pharmaceuticals Institute Co., Ltd. Kumiko Kuwaba, Kazunori Mizuno, Yuki Taga, and Masashi Kusubata were employees of Nippi, Inc. The other authors declare no competing interests.

Data Availability: The processed RNA-seq gene expression datasets and sample metadata generated and analysed during the current study are publicly available in the Zenodo repository (*30*) at https://doi.org/10.5281/zenodo.21799087. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Received August 26, 2026; Revised October 14, 2026; Accepted October 16, 2026.

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Released online in J-STAGE as advance publication October 29, 2026.

Brief Report

DOI: 10.5582/ddt.2026.01065

Patients' perceptions of hypnotic medications and factors associated with the desire to discontinue among regular users: A cross-sectional questionnaire survey in community pharmacies

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SUMMARY: Long-term use of hypnotic medications is associated with adverse events, and clinical guidelines recommend periodic reassessment and deprescribing when appropriate. However, little is known about patients who perceive hypnotic medications as necessary while wishing to discontinue treatment. This study investigated the association between perceived necessity and the desire to discontinue treatment and factors associated with discontinuation desire among patients who perceived hypnotics as necessary. A cross-sectional anonymous questionnaire survey was conducted among patients receiving regular hypnotic therapy at community pharmacies in June 2026. The questionnaire assessed perceived necessity, desire to discontinue, concerns about adverse effects, sleep quality, and treatment satisfaction. The association between perceived necessity and discontinuation desire was evaluated using the chi-square test. Factors associated with discontinuation desire among patients who perceived hypnotics as necessary were examined using multivariable logistic regression. Among 193 patients, 163 (84.5%) perceived hypnotic medications as necessary every day; of these, 56 (34.4%) wished to discontinue treatment. In contrast, 19 of 30 patients (63.3%) who did not perceive hypnotic medications as necessary every day expressed a desire to discontinue treatment ($p = 0.003$). Among patients perceiving hypnotics as necessary, concerns about adverse effects were associated with discontinuation desire (odds ratio, 4.01; 95% confidence interval, 1.92–8.38; $p < 0.001$). These exploratory findings demonstrate that perceived necessity and discontinuation desire can coexist and warrant prospective investigation of patients' perceptions and subsequent deprescribing behavior.

Keywords: Insomnia, hypnotic medications, patient perceptions, community pharmacy, adverse effects

1. Introduction

Insomnia is one of the most common sleep disorders among older adults and is an important health concern associated with reduced quality of life (QOL), impaired daytime functioning, depressive symptoms, cognitive impairment, and an increased risk of falls (*1-3*). Pharmacological treatment for insomnia includes benzodiazepines (BZs), non-benzodiazepines (Non-BZs), melatonin receptor agonists, and dual orexin receptor antagonists (DORAs), all of which have demonstrated efficacy in improving sleep symptoms (*4*).

However, long-term use of hypnotic medications has been associated with various adverse outcomes, including falls, cognitive impairment, delirium, and drug dependence, particularly among older adults because of age-related changes in pharmacokinetics and pharmacodynamics (*5-7*). Therefore, both Japanese and international clinical guidelines recommend avoiding unnecessary long-term use of hypnotics and periodically reassessing their efficacy and safety to determine whether dose reduction or discontinuation should be considered (*8-10*).

We previously reported, using the National Database of Health Insurance Claims and Specific Health Checkups of Japan (NDB) Open Data, that the use of DORAs has increased rapidly in recent years, whereas BZs and Non-BZs continue to be widely prescribed in Japan (*11*). These prescribing trends suggest that promoting the appropriate use of hypnotic medications requires not only appropriate drug selection but also a better understanding of how patients perceive their

medications.

Horne and colleagues proposed the Necessity–Concerns Framework, which suggests that patients' medication-taking behavior is influenced by the balance between their perceived necessity of treatment and their concerns about medication (*12*). A subsequent meta-analysis further demonstrated that this framework provides a useful theoretical model for understanding medication adherence across chronic diseases (*13*). More recently, a study of patients receiving hypnotic medications reported that many patients who considered hypnotics necessary nevertheless wished to reduce or discontinue treatment because of concerns regarding adverse effects and drug dependence (*14*). However, little is known about the characteristics of patients who perceive hypnotic medications as necessary while simultaneously wishing to discontinue treatment, or about the factors associated with this desire.

Therefore, the present study investigated the association between perceived necessity of hypnotic medications and the desire to discontinue treatment and factors associated with this desire among patients who perceived hypnotics as necessary.

2. Materials and Methods

2.1. Study design and participants

This cross-sectional questionnaire survey was conducted at 12 community pharmacies belonging to the Cosmo Pharma Group across eight prefectures in Japan (Hokkaido, Yamagata, Fukushima, Tochigi, Gunma, Saitama, Chiba, and Nagano). Patients who visited one of the participating community pharmacies between June 1 and June 30, 2026, and who were receiving regular treatment with hypnotic medications were eligible for inclusion. Regular hypnotic therapy was defined as having received a hypnotic prescription at two or more pharmacy visits, with at least a 14-day supply prescribed at each visit. Patients receiving hypnotic medications only on an as-needed basis were excluded. During the study period, pharmacists consecutively approached patients who met the eligibility criteria and invited them to participate in the survey. Before participation, pharmacists explained the purpose and procedures of the study, and those who agreed to participate completed an anonymous self-administered questionnaire. The total number of eligible patients approached and the number who declined participation were not recorded; therefore, an overall response rate could not be calculated.

The questionnaire was designed to be brief and easily completed during routine community pharmacy visits to minimize respondent burden. The survey assessed patients' perceptions of the necessity of hypnotic medications, desire to discontinue treatment, concerns about adverse effects, sleep quality, and satisfaction with hypnotic therapy. The exact wording of the questionnaire items and response options was as follows: (1) "Do you feel that you need sleeping medication every day?" (Yes/No); (2) "Do you want to stop taking sleeping medication?" (Yes/No); (3) "Are you concerned about the adverse effects of sleeping medication?" (Yes/No); (4) "How would you rate your overall sleep quality?" (4 = no problem [satisfied], 3 = slight problem, 2 = considerable problem, and 1 = very serious problem [dissatisfied]); and (5) "Are you satisfied with your current sleeping medication?" (4 = no problem [satisfied], 3 = slight problem, 2 = considerable problem, and 1 = very serious problem [dissatisfied]).

The question regarding the desire to discontinue treatment assessed a general desire to stop taking hypnotic medication and did not distinguish among immediate discontinuation, future discontinuation, dose reduction, or willingness to attempt deprescribing under medical supervision. The questionnaire referred to hypnotic medication use in general rather than to a specific medication. For patients receiving more than one hypnotic medication, the questionnaire did not identify whether responses referred to all hypnotics or to a specific medication.

Demographic information, including age and sex, and information on currently prescribed hypnotic medications were also collected. Hypnotic medications were classified into three pharmacological categories: novel hypnotics (dual orexin receptor antagonists and melatonin receptor agonists), non-benzodiazepines (Non-BZs), and benzodiazepines (BZs). Patients receiving more than one hypnotic medication were counted in each applicable pharmacological category.

This study was approved by the Ethics and Clinical Research Review Committee of Sainokuni Higashi-Omiya Medical Center (Approval No. 78). Written information describing the study was provided to all participants, and informed consent was obtained prior to participation.

2.2. Statistical analysis

Patient characteristics and questionnaire responses were summarized using descriptive statistics. First, among all patients included in the analysis, the association between perceived necessity of hypnotic medications and the desire to discontinue treatment was evaluated using Pearson's chi-square test. Subsequently, analyses were restricted to patients who answered that hypnotic medications were necessary every day. Among these patients, characteristics associated with the desire to discontinue treatment were examined by comparing the desire-to-discontinue and no-desire-to-discontinue groups, followed by multivariable logistic regression analysis. Categorical variables were analyzed using Pearson's chi-square test without continuity correction, age was compared using Student's *t*-test, and sleep quality and satisfaction with hypnotic therapy were

compared using the Mann–Whitney *U* test.

The primary multivariable logistic regression model included age, sex, concerns about adverse effects, sleep quality, and satisfaction with hypnotic therapy as explanatory variables. Sleep quality and satisfaction with hypnotic therapy were entered as ordinal scores ranging from 1 to 4, with the odds ratio (OR) representing the change in odds associated with a 1-point increase. Results are presented as ORs with 95% confidence intervals (CIs). Because some patients received more than one class of hypnotic medication, the distribution of single-class and combination therapy was examined. A sensitivity analysis was performed by adding the use of novel hypnotics, Non-BZs, and BZs as separate binary variables to the primary model. This approach allowed each patient to remain a single independent observation while accounting for the use of multiple hypnotic classes. Because treating the 4-point sleep quality and treatment satisfaction scales as linear terms assumes a constant change in the log odds across score levels, an additional sensitivity analysis was performed in which sleep quality and treatment satisfaction were each categorized as scores 1–2, 3, or 4. Scores 1 and 2 were combined because of the small number of patients in these categories.

All statistical analyses were performed using Python version 3.13.5 with SciPy version 1.17.0 and statsmodels version 0.14.6. All tests were two-sided, and a p value < 0.05 was considered statistically significant.

3. Results and Discussion

A total of 205 patients participated in the survey. Twelve questionnaires were excluded because at least one of the five questionnaire items was missing, leaving 193 patients for the final analysis. Of these participants, 81 (42.0%) were male and 112 (58.0%) were female, with a mean age of 69.9 ± 17.1 years. Regarding hypnotic medication use, 67 patients received novel hypnotics, 83 received non-benzodiazepines (Non-BZs), and 61 received benzodiazepines (BZs). Overall, 175 patients (90.7%) received a single hypnotic class and 18 (9.3%) received two classes; none received all three. Single-class therapy comprised novel hypnotics (n = 54), Non-BZs (n = 68), and BZs (n = 53), while combination therapy comprised novel hypnotics plus Non-BZs (n = 10), novel hypnotics plus BZs (n = 3), and Non-BZs plus BZs (n = 5). Overall, 163 patients (84.5%) reported that hypnotic medications were necessary every day, 75 (38.9%) expressed a desire to discontinue treatment, and 61 (31.6%) reported concerns about adverse effects (Table 1).

The association between perceived necessity of hypnotic medications and the desire to discontinue treatment is shown in Table 2. Among the 163 patients who reported that hypnotic medications were necessary every day, 56 (34.4%) wished to discontinue treatment. In contrast, among the 30 patients who did not perceive hypnotic medications as necessary every day, 19 (63.3%) wished to discontinue treatment. There was a significant association between perceived necessity of hypnotic medications and the desire to discontinue treatment (p = 0.003).

We next compared patient characteristics and questionnaire responses between patients who wished to discontinue treatment (desire-to-discontinue group, n = 56) and those who did not (no-desire-to-discontinue group, n = 107) among the 163 patients who perceived hypnotic medications as necessary every day (Table 3). The proportion of patients expressing concerns about adverse effects was significantly higher in the desire-to-discontinue group than in the no-desire-to-discontinue group. In addition, the use of Non-BZs was significantly more frequent, whereas the use of BZs was significantly less frequent, in the desire-to-discontinue group. No significant differences were observed between the two groups with respect to age, sex, use of novel hypnotics, or sleep quality. Satisfaction with hypnotic therapy was

Table 2. Association between perceived necessity of hypnotic medications and the desire to discontinue treatment

	Desire to discontinue		p value
	Yes	No	
Perceived hypnotics as necessary every day			0.003
Yes	56	107	
No	19	11	

P values were calculated using the chi-square test.

Table 1. Patient characteristics and perceptions of hypnotic medications

Sex	Male:81, Female:112
Age (years)	69.9 ± 17.1
Hypnotic medication class (patients receiving multiple medications were counted in each applicable class)	
Novel hypnotics	67
Non-benzodiazepines (Non-BZs)	83
Benzodiazepines (BZs)	61
Perceived hypnotics as necessary every day	163
Desired to discontinue hypnotic medications	75
Concerned about adverse effects of hypnotic medications	61
Sleep quality	3.39 ± 0.72
Satisfaction with hypnotic therapy	3.56 ± 0.64

slightly lower in the desire-to-discontinue group (p = 0.0499).

In the primary multivariable logistic regression analysis, concerns about adverse effects remained associated with the desire to discontinue treatment after adjustment for age, sex, sleep quality, and satisfaction with hypnotic therapy (OR, 4.01; 95% CI, 1.92–8.38; $p < 0.001$) (Table 4). In a sensitivity analysis additionally adjusting for the use of novel hypnotics, Non-BZs, and BZs as separate binary variables, the association between concerns about adverse effects and the desire to discontinue treatment remained statistically significant (OR, 5.06; 95% CI, 2.24–11.46; $p < 0.001$). These findings indicate that the observed association was robust to additional adjustment for hypnotic medication class. In an additional sensitivity analysis, sleep quality and satisfaction with hypnotic therapy were categorized as scores 1–2, 3, or 4 rather than entered as linear terms. Concerns about adverse effects remained associated with the desire to discontinue treatment (OR, 4.09; 95% CI, 1.95–8.60; $p < 0.001$), whereas no statistically significant associations were observed across the categories of sleep quality or satisfaction with hypnotic therapy.

The present study showed that approximately one-third of patients who perceived hypnotic medications as necessary every day nevertheless expressed a desire to discontinue treatment. Concerns about adverse effects remained associated with this desire after adjustment for the variables included in the model, suggesting that perceived necessity and discontinuation desire may coexist.

Importantly, concerns about adverse effects were not reported by all patients who wished to discontinue treatment. Among the 56 patients who perceived hypnotic medications as necessary every day and expressed a desire to discontinue treatment, 29 reported concerns about adverse effects, whereas 27 did not. Therefore, concerns about adverse effects should not be interpreted as the sole or predominant reason for the desire to discontinue treatment. Other factors not assessed in the present survey may also contribute to patients' desire to stop taking hypnotic medications.

According to the Necessity–Concerns Framework, medication-taking behavior is influenced by the balance between perceived necessity and concerns about potential adverse consequences (*12,13*). Although the present study did not evaluate this framework using a validated instrument, our findings are consistent with the concept that necessity and concerns can coexist within the same individual.

Our findings are also consistent with those of Tully *et al.*, who reported that many patients receiving hypnotic medications wished to reduce or discontinue treatment despite perceiving these medications as necessary, and that this intention was associated with concerns regarding dependence (*14*). Although differences in

Table 3. Comparison between patients with and without the desire to discontinue treatment among those who perceived hypnotic medications as necessary every day

	Desire to discontinue (n=56)	No desire to discontinue (n=107)	*p* value
Sex	Male:17, Female:39	Male:45, Female:62	0.144
Age (years)	70.8 ± 15.3	69.8 ± 18.2	0.729
Novel hypnotics	15	38	0.259
Non-benzodiazepines (Non-BZs)	34	37	0.001
Benzodiazepines (BZs)	12	43	0.016
Concerned about adverse effects of hypnotic medications	Yes:29 (No: 27)	Yes:21 (No:86)	< 0.001
Sleep quality	3.29 ± 0.73	3.47 ± 0.69	0.094
Satisfaction with hypnotic therapy	3.43 ± 0.68	3.62 ± 0.62	0.0499

Values are presented as mean ± SD or n. *P* values were calculated using Student's *t*-test for age, the Mann–Whitney *U* test for sleep quality and satisfaction with hypnotic therapy, and Pearson's chi-square test without continuity correction for categorical variables.

Table 4. Factors associated with the desire to discontinue hypnotic medications among patients who perceived hypnotic medications as necessary every day

Variable	Primary model OR (95% CI)	*p* value	Sensitivity model OR (95% CI)	*p* value
Age (per 1-year increase)	1.01 (0.99–1.03)	0.504	1.01 (0.99–1.03)	0.358
Male	0.80 (0.38–1.67)	0.548	0.66 (0.30–1.45)	0.300
Concerned about adverse effects	4.01 (1.92–8.38)	< 0.001	5.06 (2.24–11.46)	< 0.001
Sleep quality (per 1-point increase)	0.85 (0.48–1.51)	0.576	0.79 (0.43–1.47)	0.465
Satisfaction with hypnotic therapy (per 1-point increase)	0.88 (0.46–1.66)	0.685	0.94 (0.47–1.86)	0.851
Novel hypnotic use	—	—	0.39 (0.10–1.53)	0.179
Non-BZ use	—	—	1.23 (0.32–4.70)	0.764
BZ use	—	—	0.22 (0.05–0.98)	0.047

OR, odds ratio; CI, confidence interval; Non-BZ, non-benzodiazepine; BZ, benzodiazepine. The primary model included age, sex, concerns about adverse effects, sleep quality, and satisfaction with hypnotic therapy. The sensitivity model additionally included novel hypnotic, Non-BZ, and BZ use as separate binary variables. Patients receiving more than one hypnotic class were coded as users of each applicable class.

study populations and survey methods preclude direct comparison, both studies suggest that perceived necessity and the desire to discontinue treatment are not mutually exclusive. Notably, our study extends these previous findings by demonstrating that approximately one-third of community pharmacy patients who considered hypnotic medications necessary nevertheless wished to discontinue treatment and by identifying an association between concerns about adverse effects and the desire to discontinue treatment.

In the univariate analysis, Non-BZ use was more frequent and BZ use was less frequent among patients who wished to discontinue treatment. Because some patients received medications from more than one hypnotic class, we additionally performed a sensitivity analysis in which novel hypnotic, Non-BZ, and BZ use were entered as separate binary variables. The association between concerns about adverse effects and the desire to discontinue treatment remained statistically significant after additional adjustment for these medication variables. Although BZ use was also statistically associated with lower odds of expressing a desire to discontinue treatment in this sensitivity analysis, this finding should be interpreted cautiously given the limited sample size, the small number of patients receiving combination therapy, and the exploratory nature of the analysis. Further studies with larger samples and more detailed information on individual medications, doses, and treatment duration are needed to clarify the relationship between hypnotic medication class and patients' desire to discontinue treatment. Age, sex, sleep quality, and satisfaction with hypnotic therapy were not statistically associated with the desire to discontinue treatment in the multivariable analysis. However, differences in statistical significance should not be interpreted as indicating that concerns about adverse effects are more important than sleep quality or treatment satisfaction. The present study was not designed to compare the relative importance of these factors.

Long-term use of benzodiazepine receptor agonists, including both benzodiazepines and non-benzodiazepines, has been associated with daytime sedation, falls, fractures, cognitive impairment, and other adverse outcomes (*15*). Evidence-based deprescribing guidelines recommend gradual tapering, particularly in older adults, while emphasizing the importance of involving patients in shared decision-making and ensuring that they understand the rationale for deprescribing (*16*). Furthermore, recent clinical practice guidelines for insomnia recommend continuous evaluation of treatment effectiveness and safety while considering patient characteristics and preferences when selecting pharmacological therapy (*4*). Accordingly, the presence of a desire to discontinue treatment should not automatically lead to medication discontinuation. Instead, healthcare professionals should comprehensively evaluate the patient's clinical condition, treatment response, adverse effects, duration of therapy, and severity of insomnia before deciding whether to continue, modify, taper, or discontinue hypnotic medications.

Community pharmacies may represent an appropriate setting for identifying patients with ambivalent attitudes toward hypnotic medications because pharmacists have repeated opportunities to interact with patients receiving long-term pharmacotherapy. In routine pharmaceutical care, pharmacists may therefore benefit from assessing three complementary aspects: (1) whether patients perceive hypnotic medications as necessary, (2) whether they have concerns about adverse effects, and (3) whether they wish to reduce or discontinue treatment. Importantly, perceiving hypnotic medication as necessary does not necessarily indicate a willingness to continue treatment. Exploring these three perspectives and sharing information with prescribing physicians may facilitate patient-centered shared decision-making regarding continued treatment or deprescribing. Previous studies have also emphasized the importance of collaboration among patients, physicians, pharmacists, and other healthcare professionals in deprescribing benzodiazepine receptor agonists (*17*).

This study has several limitations. First, this was a cross-sectional study conducted at community pharmacies belonging to a single pharmacy group in Japan. Because the study population and practice settings were limited, caution is warranted when generalizing the findings to other populations. In addition, the total number of eligible patients and the number who declined participation were not recorded; therefore, the overall response rate could not be determined, and potential selection bias could not be fully assessed. Moreover, the cross-sectional design precludes any inference regarding causal relationships between concerns about adverse effects and the desire to discontinue treatment. Second, the questionnaire assessed patients' perceptions using single-item questions rather than validated instruments. In particular, the question regarding the desire to discontinue treatment assessed only a general desire to stop taking hypnotic medication and did not distinguish among immediate discontinuation, future discontinuation, dose reduction, or willingness to attempt deprescribing under medical supervision. In addition, for patients receiving more than one hypnotic medication, the questionnaire did not identify whether their responses referred to all hypnotics or to a specific medication. Therefore, the findings regarding the desire to discontinue treatment should be interpreted as reflecting a general patient-reported preference rather than an intention to undertake a specific deprescribing strategy. Third, several potentially important factors that may be associated with patients' desire to discontinue hypnotic treatment were not assessed in this study, including treatment duration, hypnotic dose and frequency of use, severity of insomnia, physical and psychiatric comorbidities, previous adverse effects, and previous

attempts to reduce or discontinue hypnotic medications. Therefore, residual confounding by these unmeasured factors cannot be excluded, and the associations observed in the multivariable analysis should be interpreted as adjusted only for the variables included in the present study. Future studies incorporating more detailed clinical and treatment-related information are needed to confirm these findings. Furthermore, we did not investigate the specific reasons underlying patients' desire to discontinue treatment or the nature of their concerns regarding adverse effects. Future multicenter prospective studies using validated assessment tools are warranted to clarify changes in patients' perceptions of hypnotic medications and to evaluate the effectiveness of patient-centered deprescribing interventions.

In conclusion, a substantial proportion of patients expressed a desire to discontinue hypnotic treatment despite perceiving hypnotic medications as necessary every day. Among these patients, concerns about adverse effects were associated with the desire to discontinue treatment after adjustment for the variables included in the present analysis. However, given the cross-sectional and exploratory nature of this study, these findings do not establish why patients wished to discontinue treatment or predict whether they would subsequently reduce or discontinue hypnotic medications. These findings should therefore be considered hypothesis-generating and may help inform future prospective studies examining patients' perceptions and subsequent deprescribing behavior.

Acknowledgements

We thank all study participants.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received September 13, 2026; Revised September 27, 2026; Accepted October 18, 2026.

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Released online in J-STAGE as advance publication October 21, 2026.

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DOI: 10.5582/ddt.2026.01064

Bepirovirsen: The first antisense oligonucleotide approved to achieve a functional cure for chronic hepatitis B

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SUMMARY: Chronic hepatitis B (CHB) remains a major global health burden despite the availability of potent nucleos(t)ide analogue (NA) therapy. Although NAs effectively suppress hepatitis B virus (HBV) DNA replication and reduce the risks of cirrhosis and hepatocellular carcinoma, they have limited effects on covalently closed circular DNA and hepatitis B surface antigen (HBsAg) production, and most patients require prolonged treatment. Bepirovirsen is an antisense oligonucleotide that targets conserved HBV RNA sequences, promotes RNase H1-mediated RNA degradation, and reduces viral antigen expression and replication. In August 2026, Japan approved bepirovirsen (Hibsago) for selected adults with chronic HBV infection receiving NA therapy, representing its first global regulatory approval. Approval was supported by the phase III B-Well 1 and B-Well 2 trials, in which a functional cure after treatment withdrawal was achieved in 20% and 19% of bepirovirsen-treated patients, respectively, versus 0% with a placebo. Response rates were higher among patients with lower baseline HBsAg levels. The overall safety profile was acceptable; however, elevated alanine aminotransferase levels were observed in 6% of bepirovirsen-treated participants. Ongoing studies will further define long-term durability, broader applicability, and optimal treatment strategies.

Keywords: HBV, bepirovirsen, antisense oligonucleotide, HBsAg, functional cure

Chronic hepatitis B (CHB) is a chronic inflammatory liver disease caused by persistent infection with hepatitis B virus (HBV). Clinically, persistent positivity for hepatitis B surface antigen (HBsAg) for ≥ 6 months is a key criterion for determining if a chronic HBV infection is present. Ongoing viral replication, together with host immune-mediated liver injury, drives the progression of hepatic fibrosis, and a proportion of patients eventually develop cirrhosis, hepatic decompensation, or liver cancer (*1,2*). According to data from the World Health Organization, approximately 240 million people worldwide were living with chronic hepatitis B in 2024, with an estimated 900,000 new infections occurring annually (*3*). HBV infection was responsible for approximately 1.1 million deaths in 2024, predominantly from cirrhosis and HCC (*3*). Geographically, the Western Pacific and African regions bear the greatest burden of HBV infection worldwide (*3*). In China, the fourth national seroepidemiological survey showed that the prevalence of HBsAg among individuals age 1–69 was 5.86% in 2020, which corresponds to approximately 75 million people with chronic HBV infection nationwide and which represents a persistent, substantial disease burden (*4*).

For patients with CHB who are eligible for antiviral therapy, potent nucleos(t)ide analogues (NAs) with a high barrier to resistance are the preferred treatment and remain the cornerstone of long-term antiviral therapy (*1*). First-line NAs include entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide fumarate (*1*). These agents effectively suppress HBV DNA replication and substantially reduce the risks of cirrhosis and HCC. However, NAs primarily inhibit viral reverse transcription and have little effect on covalently closed circular DNA (cccDNA) persisting in the hepatocyte nucleus. They also do not eliminate viral antigen production from integrated HBV DNA. Consequently, their effect on HBsAg levels is limited, with an annual HBsAg clearance rate of only approximately 1% during treatment (*5*). HBsAg loss remains the most desirable endpoint for discontinuing treatment, so sustained suppression of HBV DNA alone is not generally sufficient to safely discontinue therapy. Most patients therefore require long term or even lifelong treatment. There is still an urgent need for new therapeutic approaches that can reduce viral antigen levels within a finite treatment course, restore immune control, and increase the likelihood of a functional cure.

Bepirovirsen is an antisense oligonucleotide originally discovered by Ionis Pharmaceuticals and subsequently licensed to GSK for clinical development. On August 24, 2026, the Japanese Ministry of Health, Labour and Welfare approved bepirovirsen under the trade name Hibsago for adults with chronic HBV infection who had received NA therapy for ≥ 6 months and who had baseline HBsAg levels ≤ 3,000 IU/mL and HBV DNA levels < 90 IU/mL, representing the first regulatory approval of the drug worldwide (*6,7*). Bepirovirsen binds to a conserved sequence shared by multiple HBV messenger RNAs and pregenomic RNA, thereby recruiting RNase H1 to cleave and degrade the target RNAs (*8*). This mechanism simultaneously reduces viral protein expression and the generation of templates required for viral replication, leading to a reduction in levels of HBsAg, HBeAg, and HBV DNA (*8*). In addition, changes in proteins associated with innate immune responses have been observed during treatment, suggesting that bepirovirsen may also have immunomodulatory effects, potentially through Toll-like receptor 8 activation (*9,10*). Unlike NAs, which act mainly by inhibiting reverse transcription, bepirovirsen directly reduces viral RNA and antigen production. The clinical regimen consists of 24 weeks of subcutaneous bepirovirsen administered in combination with ongoing NA therapy, and it seeks to achieve a functional cure after withdrawal of all antiviral treatment. Importantly, a functional cure does not imply a sterilizing cure, as residual cccDNA may persist in hepatocytes.

Bepirovirsen's approval was substantiated by two identically designed global phase III trials, B-Well 1 and B-Well 2 (NCT05630807 and NCT05630820) (*11*). Together, the two studies enrolled more than 1,800 adults without cirrhosis from 29 countries who were receiving stable NA therapy and who had baseline HBsAg levels of > 100 to 3,000 IU/mL. Participants were randomly assigned in a 2:1 ratio to receive subcutaneous bepirovirsen at a dose of 300 mg once weekly for 24 weeks or a placebo (*11*). At week 48, NA therapy was discontinued for participants who met prespecified criteria. The primary endpoint, assessed at week 72, was a functional cure, defined as HBsAg loss together with sustained HBV DNA below the lower limit of quantification for at least 24 weeks after discontinuation of all treatment. The primary endpoint was achieved in 20% of participants in the bepirovirsen group versus 0% in the placebo group in B-Well 1 (127/650 *vs* 0/328), and in 19% of participants in the bepirovirsen group versus 0% in the placebo group in B-Well 2 (106/570 *vs* 0/286) (*11*). Among the participants with baseline HBsAg levels ≤ 1,000 IU/mL, the pooled functional cure rate increased to 26% (*11*). During the treatment period, adverse events occurred in 91% of participants in the bepirovirsen group versus 73% in the placebo group, serious adverse events occurred in 7% of participants in the bepirovirsen group versus 4% in the placebo group, and grade ≥ 3 adverse events occurred in 16% of participants in the bepirovirsen group versus 3% of participants in the placebo group (*11*). The most common grade ≥ 3 event was an increase in alanine aminotransferase levels, which occurred in 6% of bepirovirsen-treated participants (*11*). Several limitations should be considered when interpreting these findings. The trials enrolled only adults without cirrhosis in whom virological suppression had already been achieved and who had relatively low HBsAg levels, and the off-treatment observation period was limited to 24 weeks (*12*). The studies therefore do not establish the long-term risk of virological or clinical relapse, the extent of cccDNA clearance, or whether treatment translates into a reduction in cirrhosis, HCC, or mortality. Moreover, a functional cure was not achieved in approximately 80% of treated patients, and heterogeneity in treatment response and safety concerns such as elevated liver enzymes warrant further investigation.

Currently, multiple follow-up studies are underway to further strengthen the clinical evidence for bepirovirsen. B-Sure (NCT04954859) is evaluating the long-term durability of treatment response (*13*), B-Focus (NCT06497504) is assessing bepirovirsen in individuals with HIV/HBV coinfection (*14*), and B-United (NCT06537414) is investigating a sequential treatment strategy in which daplusiran/tomligisiran is followed by bepirovirsen (*15*). These studies may help define the patient populations most likely to benefit, optimize combination or sequential treatment strategies, and clarify the feasibility of sustained treatment-free disease control. In summary, bepirovirsen has demonstrated clinically meaningful efficacy with an overall manageable safety profile in carefully selected populations under controlled trial conditions. Its effectiveness in broader patient populations, long-term safety, and impact on clinically meaningful outcomes still need to be established through post-marketing and real-world studies.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received September 5, 2026; Accepted September 9, 2026.

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Released online in J-STAGE as advance publication September 25, 2026.

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DOI: 10.5582/ddt.2026.01067

Daraxonrasib: The first-in-class RAS(ON) multi-selective inhibitor approved for treatment of metastatic pancreatic adenocarcinoma

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SUMMARY: Advanced pancreatic ductal adenocarcinoma (PDAC) has a poor prognosis, with limited benefits from conventional chemotherapy. Daraxonrasib is an oral, noncovalent RAS(ON) multi-selective inhibitor that forms a ternary complex with cyclophilin A and GTP-bound RAS, blocking downstream signaling across multiple mutant and wild-type RAS proteins. On August 26, 2026, the US Food and Drug Administration approved daraxonrasib for adults with metastatic pancreatic adenocarcinoma previously treated with systemic therapy or ineligible for multiagent systemic therapy, without a mutation-specific restriction. In the randomized phase III RASolute 302 trial involving 500 previously treated patients with a good performance status, daraxonrasib improved median overall survival from 6.7 to 13.2 months (hazard ratio, 0.40) and progression-free survival from 3.6 to 7.2 months in the overall randomized population. Grade 3 or higher treatment-related adverse events were less frequent with daraxonrasib than chemotherapy (43.6% versus 57.5%), although one treatment-related death from pneumonitis occurred. Common toxicities included rash, diarrhea, and stomatitis. Limited follow-up and small non-G12 subgroups warrant caution regarding the generalizability of that trial's results. Acquired resistance, frequently involving RAS pathway reactivation, remains an important challenge. Ongoing studies are evaluating earlier treatment settings and combination strategies, while long-term safety and outcomes in patients with a poorer performance status require further investigation.

Keywords: pancreatic ductal adenocarcinoma, RAS, daraxonrasib, RASolute 302, pneumonitis

Pancreatic cancer encompasses malignancies arising from the pancreas, with pancreatic ductal adenocarcinoma (PDAC) being the most common histological subtype. Because early clinical manifestations are nonspecific, most patients present with locally advanced or metastatic disease, and approximately 80% are not candidates for upfront surgical resection at diagnosis (*1*). Data from the US Surveillance, Epidemiology, and End Results (SEER) program indicate that approximately 51% of patients have distant metastases at presentation (*2*). GLOBOCAN 2024 estimated approximately 531,300 new cases of pancreatic cancer and 490,800 associated deaths worldwide, making it the sixth leading cause of cancer-related death (*3*). In China, the National Cancer Center estimated that there were approximately 118,700 new cases and 106,300 deaths in 2022 (*4*). These estimates underscore the substantial burden of pancreatic cancer and highlight the importance of earlier diagnosis and more effective systemic therapies in improving patient outcomes.

Chemotherapy has long been the mainstay of systemic treatment for advanced PDAC. Standard first-line regimens for metastatic PDAC include FOLFIRINOX, NALIRIFOX, and gemcitabine plus nab-paclitaxel (GnP) (*5-8*). In the event of progression following first-line therapy, second-line options are selected according to prior treatment and performance status and include liposomal irinotecan plus fluorouracil and leucovorin (5-FU/LV), as well as gemcitabine-based regimens (*9,10*). Outcomes with conventional second-line chemotherapy have historically remained poor, with a median overall survival (OS) of approximately 6 months in several representative randomized clinical trials (*9,11,12*). Limited survival benefits, drug resistance, and cumulative toxicity highlight the need for new therapeutic strategies targeting the molecular drivers of tumor growth. More than 90% of PDACs harbor *RAS* mutations, predominantly involving *KRAS*, with G12D being the most prevalent variant (*13*). RAS was long considered largely inaccessible to direct pharmacological inhibition. In 2021, sotorasib became the first direct KRAS inhibitor approved by the US Food and Drug Administration (FDA), followed by adagrasib in 2022. Both agents covalently bind to KRAS G12C and stabilize it in the GDP-bound, inactive (OFF) state, and both were initially approved for previously treated *KRAS* G12C-mutated non-small cell lung cancer (NSCLC) (*14,15*). Other inhibitors in this class, including fulzerasib,

garsorasib, and glecirasib, were subsequently approved in China. However, *KRAS* G12C mutations occur in only approximately 1%–2% of pancreatic cancers, and these inhibitors do not target the more common G12D and G12V variants, leaving the targeted treatment needs of most patients with PDAC unmet (*16*).

On August 26, 2026, the FDA approved daraxonrasib (RMC-6236; RASONQUE), developed by Revolution Medicines, for adults with metastatic pancreatic adenocarcinoma who have received at least one prior systemic therapy or are ineligible for multiagent systemic therapy, without restriction to a specific *RAS* mutation (*17,18*). Daraxonrasib is an oral, noncovalent, RAS(ON) multi-selective inhibitor that binds to cyclophilin A and subsequently forms a ternary complex with GTP-bound RAS (*18*). This complex prevents RAS from interacting with downstream effectors, including RAF, thereby inhibiting RAF–MEK–ERK signaling (*19*). Unlike KRAS G12C(OFF) inhibitors, daraxonrasib directly targets the active state of RAS and exhibits inhibitory activity against wild-type and multiple mutant forms of KRAS, NRAS, and HRAS (*18*). Its activity encompasses common PDAC-associated variants, including G12D, G12V, and G12R, providing broader molecular coverage (*18,19*). Nevertheless, the absence of a mutation-specific restriction in the approved indication should not be interpreted as evidence of equivalent clinical benefit across *RAS* genotypes. Molecular profiling remains clinically important for defining tumor genotype and identifying additional molecular alterations that may inform treatment selection (*20*).

RASolute 302 (NCT06625320), an international, multicenter, open-label, phase III trial, enrolled 500 patients with metastatic PDAC who had experienced disease progression after first-line systemic therapy and had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1 (*18,20*). Patients were randomly assigned in a 1:1 ratio to receive daraxonrasib at an oral dose of 300 mg once daily or investigator-selected standard chemotherapy (mFOLFIRINOX, GnP, FOLFOX, or nal-IRI+5-FU/LV). The co-primary endpoints were OS and progression-free survival (PFS) in the *RAS* G12-mutant population, with the corresponding outcomes in the overall randomized population (*RAS* G12, G13, or Q61 mutations or with no *RAS* mutation identified) serving as key secondary endpoints (*20*). At a median follow-up of 8.5 months, median OS in the *RAS* G12-mutant population was 13.2 months with daraxonrasib versus 6.6 months with chemotherapy (hazard ratio (HR), 0.40), and median PFS was 7.3 versus 3.5 months (HR, 0.45). In the overall randomized population, median OS was 13.2 versus 6.7 months (HR, 0.40), and median PFS was 7.2 versus 3.6 months (HR, 0.49). All of those figures were statistically significant. Confirmed objective response rates in the *RAS* G12-mutant population were 33.2% in the daraxonrasib group and 11.8% in the chemotherapy group (*20*). These findings demonstrate that daraxonrasib significantly improves survival compared to standard second-line chemotherapy in patients with metastatic PDAC who have a good performance status and disease progression after first-line systemic therapy.

In the safety population, grade 3 or higher treatment-related adverse events occurred in 43.6% of patients receiving daraxonrasib and 57.5% of those receiving chemotherapy (*21*). Treatment-related adverse events led to permanent treatment discontinuation in 1.2% and 11.2% of patients, respectively (*21*). Grade 3 or higher adverse events of any cause occurred in 61.8% and 69.6% of patients, respectively (*20,21*). Common treatment-related adverse events associated with daraxonrasib included rash, diarrhea, and stomatitis, and one patient died from treatment-related pneumonitis (*18*). Thus, despite lower rates of high-grade treatment-related adverse events and treatment discontinuation than with chemotherapy, daraxonrasib-associated toxicities warrant careful clinical attention. Additional limitations include the open-label design, relatively short follow-up, and small number of patients without *RAS* G12 mutations (8%), including only 3% patients with no detectable RAS mutation (*18*). These factors limit the certainty of efficacy estimates in the respective subgroups. Because the trial enrolled only patients with an ECOG performance status of 0 or 1, the benefit–risk profile in patients with a poorer performance status remains to be established.

The ongoing phase III RASolute 303 trial is comparing daraxonrasib monotherapy, daraxonrasib plus GnP, and GnP alone as first-line treatment (*22*). RASolute 304 is evaluating daraxonrasib versus standard observation following surgical resection and completion of neoadjuvant and/or adjuvant chemotherapy, whereas RASolve 301 is comparing daraxonrasib with docetaxel in treating previously treated *RAS*-mutated NSCLC (*23,24*). These studies aim to further define the role of daraxonrasib across treatment settings and in other *RAS*-driven malignancies, and its value as first-line or adjuvant therapy remains to be established. Although randomized evidence has demonstrated a survival benefit for daraxonrasib, its effectiveness in routine clinical practice, long-term safety, and benefits in specific patient populations require further evaluation. Acquired resistance is another important concern. Secondary mutations at KRAS residue Y64 have been reported in patients with other solid tumors and can confer resistance by disrupting ternary complex formation (*25*). In contrast, a clinical resistance analysis involving 44 patients with PDAC identified no acquired secondary *KRAS* mutations; more frequently observed alterations included *KRAS* amplification and aberrant downstream or bypass signaling (*26*). These findings suggest that mechanisms of resistance to daraxonrasib may differ across tumor types. Further elucidation of resistance mechanisms, identification of associated biomarkers, and development of mechanistically informed combination strategies will be important for optimizing its clinical use.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received September 16, 2026; Accepted September 19, 2026.

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Released online in J-STAGE as advance publication September 25, 2026.

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DOI: 10.5582/ddt.2026.01082

Rebisufligene etisparvovec: The first AAV9-mediated *SGSH* gene therapy approved for the treatment of mucopolysaccharidosis type IIIA (MPS IIIA)

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SUMMARY: Mucopolysaccharidosis type IIIA (MPS IIIA; Sanfilippo syndrome type A) is a rare lysosomal storage disorder caused by biallelic *SGSH* pathogenic variants that results in heparan sulfate accumulation and progressive neurodegeneration. Previously, treatment was limited to supportive care. In September 2026, the US Food and Drug Administration approved rebisufligene etisparvovec-hopf (FAYUVI), an adeno-associated virus serotype 9–mediated *SGSH* gene-addition therapy, for neurological manifestations in pediatric patients with preserved neurodevelopmental function. Evidence was primarily derived from the open-label Transpher A study, which compared 17 early-treated patients with 27 external natural history controls. Between 24 and 60 months of age, the model-estimated difference in the change in Bayley-III cognitive raw score was 23.5 points (95% confidence interval, 17.2–29.9), favoring treatment. Heparan sulfate levels in cerebrospinal fluid decreased by a mean of 57.1% at 24 months. Among patients receiving the recommended dose, elevated liver enzymes (85%) and vomiting (67%) were common; serious adverse events occurred in 18 of 33 patients across all dose levels. The approval of rebisufligene etisparvovec represents a landmark advance in the treatment of MPS IIIA, establishing the first disease-modifying gene therapy for this disorder and demonstrating the therapeutic potential of early intervention. Ongoing follow-up and postmarketing surveillance need to be conducted to establish the therapy's long-term efficacy, durability, and safety.

Keywords: mucopolysaccharidosis, Sanfilippo syndrome, rebisufligene etisparvovec, gene therapy, heparan sulfate

Mucopolysaccharidosis type IIIA (MPS IIIA), also known as Sanfilippo syndrome type A, is an autosomal recessive lysosomal storage disorder. Biallelic pathogenic variants in the *SGSH* gene result in a deficiency of N-sulfoglucosamine sulfohydrolase (sulfamidase), impairing the degradation of heparan sulfate (HS) and leading to its accumulation in tissues and progressive neurodegeneration (*1*). Affected individuals gradually lose cognitive, language, and motor abilities and develop behavioral disturbances, sleep disorders, and swallowing difficulties. As the disease progresses, patients often become dependent on comprehensive care, placing a substantial long-term burden on their families. Premature death may occur due to neurologic disease-related complications (*1,2*).

A global systematic review and meta-analysis estimated the pooled birth prevalence of MPS IIIA to be approximately 0.52 per 100,000 live births (95% confidence interval [CI], 0.33–0.72), although estimates vary across regions because of differences in diagnostic capabilities and epidemiological methods (*3*). In children with rapidly progressive disease, cognitive development typically reaches a plateau at approximately 30 months of age, followed by marked regression at 40-50 months, suggesting that the window for effective intervention occurs early in life (*1*). Currently, the management of MPS IIIA relies primarily on supportive and symptomatic care, while established treatments capable of modifying disease progression have historically been lacking. There is therefore an urgent need for disease-modifying therapies that can be administered before the onset of cognitive decline.

On September 17, 2026, the US Food and Drug Administration (FDA) approved rebisufligene etisparvovec (FAYUVI; formerly UX111/ABO-102), developed by Ultragenyx, for the treatment of neurological manifestations of MPS IIIA in pediatric patients with preserved neurodevelopmental function, making it the first FDA-approved treatment for this disease (*4*). Rebisufligene etisparvovec uses an adeno-associated virus serotype 9 (AAV9) vector to deliver a functional SGSH expression cassette, thereby restoring SGSH enzymatic activity and promoting the lysosomal degradation of heparan sulfate (*5*). This gene replacement strategy provides a functional copy of the gene without correcting the underlying pathogenic variants. The recommended dose is a single intravenous

infusion of 3.0×10^{13} vector genomes/kg (*5*). The principal advantages of this approach are its ability to address the underlying enzymatic deficiency and its single-dose administration, potentially eliminating the need for repeated delivery of therapeutic agents to the central nervous system. Rebisufligene etisparvovec therefore represents a disease-modifying treatment option for a defined population of pediatric patients, and its efficacy cannot yet be extrapolated to patients in all stages of MPS IIIA.

The clinical evidence supporting its approval was derived primarily from Transpher A (ABT-001; NCT02716246), a global, open-label, single-arm, dose-escalation phase I/II/III study, together with data from its long-term follow-up study (LTFU-ABO-102; NCT04360265) (*5*). A total of 28 children with rapidly progressing MPS IIIA received treatment in the main study, including 22 who received a single intravenous infusion at the recommended dose of 3×10^{13} vector genomes/kg (*6*). Of those 22 patients, 17 met the criteria for the modified intention-to-treat (mITT) population: age ≤ 2 years, or age > 2 years with a baseline cognitive developmental quotient > 60 (*5,6*). These 17 patients constituted the primary efficacy population and were compared with 27 untreated patients from external natural history studies. The primary efficacy outcome was the change in Cognitive Scale raw scores on the Bayley Scales of Infant and Toddler Development, Third Edition (BSITD-III). The percent change in the heparan sulfate level in cerebrospinal fluid (CSF) from the baseline was evaluated as a secondary endpoint. After a median follow-up of 4.2 years in the treatment group, the model-estimated changes in cognitive raw scores on the BSITD-III between chronological ages of 24 and 60 months were + 16.0 points in treated patients and − 7.6 points in natural history controls (*5*). The between-group difference was 23.5 points (95% CI, 17.2-29.9; $P < 0.0001$), suggesting that treated patients acquired or maintained cognitive skills more than would be expected from the natural course of the disease (*5*). At 24 months after treatment, heparan sulfate levels in CSF had decreased by an average of 57.1% from the baseline among 16 evaluable patients (*5*). In a pooled safety analysis, the incidence of elevated liver enzymes was 85%, that of vomiting was 67%, and that of a decreased platelet count was 19% among 27 patients receiving the recommended dose (*6*). Elevation of liver enzymes was mild or moderate (grade 1 or 2); three patients experienced grade 3 elevation, which resolved spontaneously or following corticosteroid treatment (*6*). Across all dose levels, 18 of 33 patients experienced serious adverse events, including one episode of an altered mental status considered possibly related to treatment (*6*). No deaths were reported.

As of October 19, 2026, the expansion cohort of the Transpher A study remained open for enrollment, with ongoing efforts to optimize immunomodulatory management and gain additional clinical experience (*7*). In addition, a long-term follow-up study (NCT04360265) continued to evaluate safety and cognitive outcomes (*8*). Although the available clinical data substantiate a cognitive benefit of rebisufligene etisparvovec when administered early in the disease course and they provide an initial characterization of its safety profile, the therapy's efficacy in real-world clinical practice, the durability of its therapeutic benefit, and its long-term safety remain to be established through postmarketing registries and systematic follow-up studies.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received October 22, 2026; Accepted October 27, 2026.

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Released online in J-STAGE as advance publication October 28, 2026.

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DOI: 10.5582/ddt.2026.01086

Surgical indications for hepatocellular adenoma: Diagnostic uncertainty and molecular risk stratification

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SUMMARY: Surgical management of hepatocellular adenoma (HCA) is still guided principally by patient sex, tumor size, and growth, although molecular classification has revealed important biological differences. Retrospective studies have also exposed diagnostic discordance, complicating the interpretation of historical estimates of malignant risk. Molecular subtypes differ in their associations with hemorrhage and malignant transformation, but evidence that subtype-guided management improves outcomes remains limited. Proposals to observe selected men with small lesions have reopened the debate over routine resection. We examine how diagnostic confidence, molecular heterogeneity, and the limitations of available outcome data should inform surgical decisions. Any change in practice should be supported by validated estimates of clinical risk rather than molecular findings alone.

Keywords: hepatobiliary surgery, malignant transformation, hemorrhage risk, molecular pathology, clinical surveillance

1. Introduction

Hepatocellular adenoma (HCA) is a benign hepatocellular neoplasm that can cause hemorrhage or undergo malignant transformation. Molecular studies have clarified its heterogeneity, and yet management remains anchored in clinical risk factors. The European Association for the Study of the Liver (EASL) guidelines and the 2024 American College of Gastroenterology (ACG) guidelines recommend resection or definitive treatment in men regardless of tumor size. In women, lesion size, growth, and response to withdrawal of hormonal exposure or other risk-factor modification guide management; the ACG also emphasizes resection for β-catenin–mutated lesions irrespective of size (*1,2*).

These recommendations offer a workable clinical framework, but improved imaging and molecular characterization have made the limits of historical risk estimates more apparent.

2. Diagnostic uncertainty and the interpretation of historical risk

The recommendation for routine resection in men was influenced by Farges *et al*., who reported malignant transformation in 47% of men versus 4% of women in a tertiary-center series of 218 patients with histologically documented HCA (*3*). Those proportions describe a selected clinical cohort, not the prospective lifetime risk of malignancy in an unselected population.

More recent studies have demonstrated that diagnostic classification itself may be a major source of uncertainty. In a Dutch nationwide retrospective study of 66 men with an initial pathological diagnosis or differential diagnosis of HCA, an expert review supported by molecular analysis resulted in diagnostic reclassification in 33 patients (50%) (*4*). Importantly, the study also found clinically relevant differences in outcomes among patients ultimately classified as having HCA, uncertain HCA/hepatocellular carcinoma (HCC), or HCC.

In another nationwide study involving 222 patients undergoing resection for suspected HCA, Haring *et al*. reported postoperative diagnostic changes in 96 patients (43%). Male sex, smaller tumor size, and the absence of hepatobiliary contrast-enhanced magnetic resonance imaging were independently associated with diagnostic discordance (*5*).

The two Dutch studies used different definitions of diagnostic change and included selected patients. Reclassification should not be mistaken for malignant transformation during follow-up. Rather, these findings show how uncertainty at diagnosis may distort

Table 1. Key evidence informing the surgical indications for and risk stratification of hepatocellular adenoma

Study	Study design / population	Main findings	Implications and limitations
EASL (2016) and ACG (2024) (*1,2*)	Clinical practice guidelines	Men: resection or definitive treatment irrespective of size; women: observation after risk-factor modification for selected lesions, with the 5-cm threshold and β-catenin status guiding treatment	Current standard framework; molecularly individualized decisions remain not fully validated
Farges *et al.* (2011) (*3*)	Retrospective tertiary-center study; 218 histologically confirmed cases	Malignant transformation reported in 47% of men and 4% of women in this selected cohort	Influential historical evidence; selected population limits generalization
van Rosmalen *et al.* (2021) (*4*)	Nationwide retrospective pathological review; 66 men	Diagnosis reclassified in 33 patients (50%)	Demonstrates diagnostic uncertainty; selected pathology-registry population
Haring *et al.* (2023) (*5*)	Nationwide surgical cohort; 222 patients	Postoperative diagnostic change in 96 patients (43%)	Demonstrates discordance in a surgical cohort; cannot estimate population-wide error or malignant transformation rates
Nault *et al.* (2017) (*6*)	Multicenter molecular analysis; 533 HCAs from 411 patients	Molecular subtypes had different associations with bleeding and malignant transformation	Foundational biological evidence; not a prospectively validated treatment algorithm
Sempoux and Park (2026) (*7*)	Molecular pathology review	Updated classification integrates morphology and molecular characteristics	Improved diagnostic framework; direct treatment implications require outcome validation
Bioulac-Sage *et al.* (2026) (*8*)	Pathological series; 17 patients with resected sonic hedgehog HCA	Hemorrhage and microscopic vascular injury were also observed in small lesions	Supports size-independent biological features; does not quantify prospective clinical bleeding risk
Bioulac-Sage *et al.* (2026) (*9*)	Selected multicenter pathological series; 26 patients	Distinct HCA molecular subtypes coexisted within individual patients	Highlights inter-tumor heterogeneity; prevalence cannot be inferred from this selected series
Belledent *et al.* (2026) (*10*)	Narrative review focused on men	Proposes consideration of surveillance for selected men with lower-risk lesions	Emerging proposal, not a new guideline or prospectively proven standard

This table is a critical narrative synthesis of selected studies rather than a systematic review or quantitative meta-analysis. All numerical results refer to their respective study populations. *Abbreviations*: ACG, American College of Gastroenterology; EASL, European Association for the Study of the Liver; HCA, hepatocellular adenoma; HCC, hepatocellular carcinoma.

estimates of the risk attributed to HCA.

Before changing management on the assumption that a lesion is benign, clinicians must therefore consider how confidently HCA has been distinguished from well-differentiated HCC and other hepatocellular lesions.

3. Molecular heterogeneity and distinct clinical risks

In a multicenter analysis of 533 HCAs from 411 patients, Nault *et al*. linked molecular subtypes to patient characteristics, bleeding, and malignant transformation (*6*). Sempoux and Park subsequently described five principal molecular subtypes within the updated WHO framework, integrating morphology, immunohistochemistry, and molecular alterations (*7*).

The clinical consequences differ by subtype. Strongly activating CTNNB1 exon 3 alterations are associated with greater malignant potential, whereas sonic hedgehog–activated HCA and some CTNNB1 exon 7/8–mutated lesions have been linked to bleeding (*6,7*). Hemorrhage and malignant transformation should therefore be assessed as distinct outcomes rather than grouped under an undifferentiated high-risk label.

In a pathological series of 17 patients with sonic hedgehog–activated HCA, Bioulac-Sage *et al*. identified macroscopic hemorrhage and microscopic vascular injury in both small and large nodules (*8*). These findings suggest that hemorrhage-associated tissue changes are not restricted to tumors larger than 5 cm; however, the series does not measure prospective rates of clinically significant bleeding in small lesions.

Multiplicity adds another difficulty. In a selected series of 26 patients with inter-tumor heterogeneity, Bioulac-Sage *et al*. identified different HCA subtypes in the same liver, including subtypes associated with malignant progression or hemorrhage (*9*). Molecular analysis of one nodule may therefore fail to characterize the other lesions.

Molecular classification can inform assessment but has not been prospectively validated as a replacement for size, growth, imaging findings, or clinical context. Sampling error and overlap with well-differentiated HCC also limit the reassurance that immunohistochemistry or targeted sequencing can provide.

4. Reconsidering surgical indications without prematurely changing practice

Belledent *et al*. recently questioned universal resection in men and discussed surveillance for selected, carefully characterized low-risk lesions (*10*). Avoiding surgery in such patients could be beneficial, but the proposed selection criteria and surveillance approach have not been prospectively validated.

A biopsy without a detected high-risk mutation cannot exclude an unsampled malignant focus, and long-term outcomes of molecularly characterized, unresected HCA in men remain uncertain. Selective surveillance should thus be presented as a proposal for further evaluation, not as an alternative already endorsed by current guidelines. Table 1 distinguishes established recommendations from observational evidence and recent proposals.

5. Priorities for evidence-based risk-adapted management

Prospective multicenter registries should enroll consecutive patients with newly detected HCA, not only those referred for resection. Standardized multiphasic MRI, an expert pathological review when tissue is available, and clinically justified molecular testing would allow diagnostic confidence to be recorded alongside management decisions.

Clinically significant hemorrhage and malignant transformation should be prespecified as separate outcomes. Analyses should address size, growth, molecular subtype, hormonal and metabolic exposures, background liver disease, lesion multiplicity, treatment morbidity, and patient preferences. Comparisons of surveillance with intervention will also need to address confounding by indication and changes in diagnosis over time.

The relevant question is whether molecular data improve risk prediction and treatment decisions beyond established clinical and imaging variables. Any proposed model will require external validation in different populations and care settings.

Minimally invasive techniques may reduce the burden of liver resection, but technical feasibility is not itself an indication to operate on a benign lesion. Equally, molecular refinement does not establish that observation is safe.

The priority is to determine which patients are genuinely at risk of harm and which are more likely to be harmed by intervention. Surgical indications should evolve from more reliable diagnoses and validated outcome-based risk estimates, rather than from either tumor diameter or molecular subtype alone.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received October 4, 2026; Revised October 27, 2026; Accepted October 29, 2026.

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Released online in J-STAGE as advance publication October 31, 2026.

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(As of August 2026)

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