A drug repurposing study based on clinical big data for the protective role of vitamin D in olanzapine-induced dyslipidemia

(臨床ビッグデータに基づくオランザピン誘発脂質異常症に対するビタミンDの予防作用の解明)

2022

ZHOU ZIJIAN

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Abstract

Olanzapine is one of the most used atypical antipsychotics treating schizophrenia by blocking the dopamine D_2 receptors and serotonin 5-HT_{2A} receptors in the central nervous system. However, it is reported to have a tremendous clinical risk of inducing dyslipidemia, characterized by increased triglycerides and low-density lipoproteins, accompanied by decreased high-density lipoproteins in the blood. So far, there is neither a profound understanding of the molecular mechanism of olanzapine-induced dyslipidemia nor effective treatments or interventions for the adverse events. In attempts to find a new approach, fast and low-cost drug development through drug repurposing methods has become a promising alternative strategy. Several explorations have been made in drug repurposing studies. One of them used clinical big data, which consists of an enormous collection of detailed medical practices with particular outcomes, allowing investigations on ever-overlooked drug-drug interactions and new treating potentials of the present drugs for unknown targets. This study followed this path by joining three separate sources of clinical big data for a cross-corroboration on the protective effects of vitamin D supplements on blood lipid profile deterioration that happens when treated with olanzapine. This hypothesis was validated in rodent *in vivo* models and further explored for the molecular mechanisms by *in vitro* models.

Chapter 1. Data mining approach for effective prevention of olanzapine-induced dyslipidemia

Three sources of clinical big data were utilized for large-scale screening of drugs potent to prevent olanzapine-induced dyslipidemia. First, the US Food and Drug Administration adverse event reporting system investigations revealed that vitamin D supplementary is strongly associated with a lower reporting rate of olanzapine-induced dyslipidemia. JMDC insurance claims containing precise dates of prescriptions were introduced to establish a clear causal relationship. A significant suppression by vitamin D

supplements on olanzapine-induced blood lipid profile deterioration was detected in annual blood tests. Furthermore, daily tracing of blood lipid profiles in the clinical data warehouse medical records of Nihon University School of Medicine's found that olanzapine induces dyslipidemia as early as six months after the first prescription while vitamin D constantly maintained blood lipid level stable. To conclude, vitamin D supplements have clinical significance in preventing olanzapine-induced dyslipidemia.

Chapter 2. Experimental validation for the effect of vitamin D on olanzapine-induced dyslipidemia

A rodent model of olanzapine-induced dyslipidemia was established in mice administrated with olanzapine (10 mg/kg) orally for five days. The blood test on mice after the treatment displayed increased low-density lipoproteins and decreased high-density lipoproteins, implicating a disturbance of blood lipid homeostasis by olanzapine. A one-week preload of a vitamin D-supplemented diet significantly improved these changes. Further investigations on the direct impacts of olanzapine and vitamin D on cholesterol biosynthesis in cultured mice cells with RNA-seq revealed that olanzapine hardly directly influenced the cholesterol synthesis process. In contrast, $1,25(OH)_2D_3$ (bioactive form of vitamin D) had an apparent inhibition on the cholesterol biosynthesis gene expressions in the C2C12 mouse myocyte cell line, which is further proved to be mediated by upregulation of *Insig2* expression via activation of vitamin D receptors.

Abbreviations

$1,25(OH)_2D_3$:	1, 25-dihydroxyvitamin D_3
$25(OH)D_3$:	25-hydroxyvitamin D_3
ATC:	anatomical therapeutic chemical
BMI:	body mass index
CI:	confidence interval
Cyp51:	cytochrome P450, family 51
Dhcr24:	24-dehydrocholesterol reductase
DMEM:	Dulbecco′s modified eagle′s medium
EDTA:	ethylenediaminetetraacetic acid
FBS:	fetal bovine serum
FDA:	Food and Drug Administration
Fdft1:	farnesyl diphosphate farnesyl transferase 1
Fdps:	farnesyl diphosphate synthetase
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
HBSS:	Hanks' balanced salt solution
HDL:	high-density lipoprotein
HEPES:	hydroxyethyl piperazineethanesulfonic acid
Hmgcr:	3-hydroxy-3-methylglutaryl-CoA reductase
Hmgcs1:	3-hydroxy-3-methylglutaryl-CoA synthase 1
HRP:	horseradish peroxidase
ICD10:	international classification of disease 10
IgG:	immunoglobulin G
Insig2:	insulin-induced gene 2
JMDC:	Japanese medical data center insurance claims database

LDL:	low-density lipoprotein
Lss:	lanosterol synthase
MedDRA:	medical dictionary for regulatory activities
MeSH:	medical subject headings
Nsdhl:	NAD(P) dependent steroid dehydrogenase-like
NUSM:	Nihon university school of medicine
qRT-PCR:	real-time quantitative reverse transcription polymerase chain reaction
RIPA	radioimmunoprecipitation
ROR:	reporting odds ratio
Rplp0:	ribosomal protein lateral stalk subunit P0
RT:	room temperture
SDS:	incidence rate ratio
SREBPs:	sterol regulatory element binding transcription factors
TBS-T:	Tris-buffered saline supplemented with 0.1% of Tween 20
VD_3:	vitamin D_3
VDR:	vitamin D receptor

Chapter 1. Data mining approach for effective prevention of olanzapine-induced dyslipidemia

1.1. Introduction

Schizophrenia includes a series of mental disorders like hallucinations, reduced motivation, delusions, and either negative or positive symptoms [1]. Currently, the most effective intervention is the treatment by atypical antipsychotics, also known as second-generation antipsychotics, which act by inhibiting the actions of dopamine D2 receptors and serotonin 5-HT_{2A} receptors [2]. Among them, olanzapine is one of the most widely applied agents [3]. However, dyslipidemia has been clinically reported to be a typical adverse effect of olanzapine treatment, characterized by symptoms such as increased body mass and increased serum levels of total cholesterol and triglycerides [4]. A recently published study on the adverse effects of olanzapine further confirmed that olanzapine could induce LDL cholesterol level increase in the blood [5]. Although olanzapine is pharmacologically considered cardiac-safe, its indirect promotion of atherosclerosis by raising blood lipid content makes it a cardiovascular risk factor [6-8]. These defects even make olanzapine a contributor to excessive premature mortality [9]. Up to now, the preliminary clarification on the mechanism behind olanzapine-induced dyslipidemia makes it impossible to develop treatment or prevention methods with clinical significance [10]. Thus, there is an emerging demand for exploring novel drug target screening approaches.

In consideration of saving time and cost, drug-repurposing is recognized as an economical approach to refine new potential therapeutic applications from market-accessible drugs [11]. Besides, without the development of new agents from unprecedented compounds, clinical safety has already been validated by historical early-stage trials in the drug-repurposing practice [12, 13].

So far, drug repurposing studies have revealed that a newly-released anticonvulsant is capable of attenuating the obesity-inducing effect of olanzapine [14]. Also, diabetes-treating drugs like metformin and liraglutide have been reported to suppress excessive body weight gain caused by olanzapine exposure [15, 16]. However, in a larger scale of clinical cohort studies, no effective interventions have been proven to be effective in atypical antipsychotics (including olanzapine)-induced dyslipidemia, according to a recently published meta-analysis based on clinical trials focusing on blood lipid [17]. Therefore, regarding the fact that existing drug repurposing approaches aiming to refine interventions with impressive clinical performance in treating olanzapine-induced dyslipidemia failed, an optimized approach is required to solve these problems.

Therefore, an updated approach, named “reverse translational drug repurposing”, is adopted in the present study [18]. Unlike previous reports using limited clinical proofs to establish the hypothesis, this approach makes it possible to conduct retrospective analysis with real-world clinical big data and screen for hypothetical drug-drug pairs that potently attenuate olanzapine-induced dyslipidemia in patients.

The first data source to introduce is the FAERS. The FAERS is a database collecting worldwide spontaneous adversary events reports, and the expressions of specific adverse effects are based on MedDRA terminology [19]. The confounding factors that potentially increase or decrease the occurrence of the adversary events induced by any specific drug included in this system can be identified. The application of FAERS in the present study provides several advantages: A wide diversity in races background, living environments, and living habits. A previous attempt from our laboratory using the self-reports of adverse events extracted from the FAERS succeeded in revealing the mechanism of hyperglycemia induced by quetiapine, another atypical antipsychotic, and found the agent

with treating potentials [20]. However, reports in FAERS lack time information to establish the precedence relationship between cofounding factors and adversary events.

To improve the performance of this approach, another source of clinical big data, insurance claims from JMDC Inc., was introduced. JMDC has been collecting medical records from health insurance providers since 2005. JMDC provides easy access to the actual period (in units of month or year) of any prescriptions, and the sequential association between drug exposure and disease onset can be clearly established in the history of a specific individual. Our team recently published studies based on the analysis of this database [21, 22]. Nevertheless, the blood test results included in JMDC databases were only collected yearly, so the precise onset time of olanzapine-induced dyslipidemia may not be correctly represented.

Therefore, in the present study, in addition to the FAERS and JMDC database, the electronic medical records from the clinical data warehouse of NUSM, containing detailed diagnostic, demographic, and laboratory data of hospitalized patients and out-patients at three affiliated hospitals with NUSM were also included [23]. Electronic medical records can provide continuous clinical records, allowing the detection of the precise onset time of the adverse effect of a specific drug in units of days. To conclude, the corroborating results from the analysis of the three data sources introduced above enable a more sensitive retrospective analysis on detecting causal relationships between the resultant adverse events and specific drug treatment or the lowered adverse event incidence by potent rescuing drugs.

1.2. Method

1.2.1. Analysis of the FAERS Database

Adversary event reports from 2004 to 2019 were obtained from the FDA website (https://www.fda.gov/drugs/drug-approvals-anddatabases/fda-adverse-

event-reporting-system-faers). Duplicated reports were removed by the method reported previously [24]. 11,438,031 preserved reports were included in the analysis of the present study. Arbitrary drug names, including trade names and abbreviations, were manually mapped to unified generic names with MeSH descriptor ID. FAERS were analyzed in terms of previously described [20]. In detail, the individuals were divided into four groups: (a) individuals who received the drug of interest (drug A) exhibited dyslipidemia; (b) individuals who received drug A but did not exhibit dyslipidemia; (c) individuals who did not receive the drug A and exhibit dyslipidemia; and (d) individuals who did not receive the drug A and did not exhibit dyslipidemia. The reporting odds ratio (ROR) with 95% CI and *Z*-score was calculated as per formulae 1 – 3:

$$ROR = \frac{a/b}{c/d} \quad \text{1}$$

$$95\%\ \text{CI} = \exp\left\{log(ROR) \pm 1.96\sqrt{\frac{1}{a}+\frac{1}{b}+\frac{1}{c}+\frac{1}{d}}\right\} \quad \text{2}$$

$$Z\ \text{score} = \frac{log(ROR)}{\sqrt{\frac{1}{a}+\frac{1}{b}+\frac{1}{c}+\frac{1}{d}}} \quad \text{3}$$

Where *a*, *b*, *c* ,and *d* refer to the number of individuals in each group and log refers to the common logarithm.

To further investigate the drugs reducing the occurrence of olanzapine-induced dyslipidemia. The individuals who received olanzapine were divided into the following four groups: (a1) individuals who received the concomitant drug of interest (drug B) and exhibited dyslipidemia; (b1) individuals who received drug B, but did not exhibit dyslipidemia; (c1) individuals who did not receive drug B and exhibited dyslipidemia; and (d1) individuals who did not receive drug B and did not exhibit dyslipidemia. The reporting odds ratio (ROR) with 95% confidence interval

(CI) and *Z*-score for olanzapine-induced dyslipidemia was calculated as per formulae 4 – 6:

$$ROR = \frac{a1/b1}{c1/d1} \quad \text{4}$$

$$95\%\ \text{CI} = \exp\left\{log(ROR) \pm 1.96\sqrt{\frac{1}{a1}+\frac{1}{b1}+\frac{1}{c1}+\frac{1}{d1}}\right\} \quad \text{5}$$

$$Z\ \text{score} = \frac{log(ROR)}{\sqrt{\frac{1}{a1}+\frac{1}{b1}+\frac{1}{c1}+\frac{1}{d1}}} \quad \text{6}$$

where *a1*, *b1*, *c1*, and *d1* refer to the number of individuals in each group, and log refers to the common logarithm.

1.2.2. Analysis of the JMDC Claims Database

Insurance claims data collected by JMDC Inc. from January 2005 to March 2018 were purchased, containing medical and prescription claims of 5,550,241 participants and their dependents on a monthly basis. Blood test results, BMI, and waist were provided by 2,278,697 of them. Patients were mainly aged ≤ 65 years, and no patients aged ≥ 75 years were included. The drug names were coded by ATC Classification System. By retrospection on the profile of the event onsets, the olanzapine users were identified as those who were prescribed olanzapine more than two months after being included in the JMDC claim database. Simultaneously, those patients without blood test results were excluded. Among them, preceding users of vitamin D were defined as patients whose first prescription of vitamin D was ahead of their olanzapine treatment starting for at least one month. The pre-prescription period was defined as within 12 months, while, in parallel, the post-prescription period was defined as within 12 months after starting olanzapine treatment. Aside from BMI and waist circumstances, the blood test results included in the present study are triglyceride, LDL cholesterol, and HDL cholesterol levels. The test results

were collected at the nearest date before olanzapine treatment starting during the pre-prescription period and on the nearest date after the olanzapine treatment starting within the post-prescription period. The propensity score matching method (greedy 1:1 matching) was applied to reduce the bias in the population backgrounds by balancing covariates between settings. The propensity score of vitamin D was obtained by fitting a logistic regression model that included all the covariates of interest (Table 1.2). After constructing the score, each individual's propensity score with or without vitamin D treatment was matched by the nearest neighbor method. With matched outcomes, exact tests for categorical data were conducted to compare differences in baseline characteristics between individuals with or without vitamin D exposure.

1.2.3. Analysis of the NUSM Electronic Medical Records

Electronic medical records were obtained from the Nihon University School of Medicine's Clinical Data Warehouse (NUSM's CDW). This database includes detailed diagnostic, demographic, and laboratory data for inpatients and outpatients. We received written informed consent and agreement for the secondary use after anonymization at three hospitals affiliated with the NUSM [23]. The pre-prescription period of olanzapine was defined as the period within 12 months before the first prescription. At the same time, the post-prescription period was defined as the period within 12 months after the start of olanzapine treatment. Among the patients using olanzapine, preceding users of vitamin D were defined as patients whose first prescription of vitamin D was ahead of the olanzapine treatment for at least one day. Matching was not performed in this study as there were no significant differences in the baseline characteristics, shown in table 1.3. The blood test results of the nearest test prior to the first prescription of olanzapine were selected as the

baseline value, expressed as Month 0. The values of each checkpoint (Months 3, 6, 9, 12) were selected from the furthest test result from the first prescription of olanzapine. Missing values were imputed by the Last observation carried forward (LOCF) method. An unpaired two-tailed *t*-test with Welch's correction for continuous variables and Fisher's exact test for categorical data were conducted to compare differences in baseline characteristics between patients with or without vitamin D exposure.

1.2.4. Statistics

Statistical analysis of the FAERS database was performed with SQL Software (IBM research laboratory, NY, USA), and that of the JMDC database was performed with R version 3.5.1 Software (The R Foundation for Statistical Computing, Vienna, Austria). Statistical analysis of the FAERS database was performed with SQL Software (IBM research laboratory, NY, USA), and that of the JMDC database was performed with R version 3.5.1 Software (The R Foundation for Statistical Computing, Vienna, Austria). In the JMDC database, paired *t*-tests were utilized to compare the mean values within pre-prescription and post-prescription periods. Differences in continuous variables between these two groups were compared via an unpaired two-tailed *t*-test with Welch's correction. In the NUSM database, mixed-effects models were used to analyze repeated measures data.

1.3. Results

1.3.1. Analysis of FAERS

Firstly, the association between the treatment of a particular and the incidence of dyslipidemia in the FAERS database was investigated using disproportionality analysis by calculating the ROR and *Z*-score. (Figure 1.1A). The known bias and the

lack of knowledge about the size of the total population only make the results of this analysis a limited demonstration of the real-world incidence rate [25]. Nevertheless, olanzapine is still revealed to be one of the drugs that exhibit the strongest association between their usage and the onset of dyslipidemia by both high ROR and *Z*-score. Olanzapine is selected for the following research due it stands a higher ROR value than any other atypical antipsychotics (Table 1.1). The confounding effects of all the drug combinations used in the olanzapine-treated patients were also calculated by ROR and *Z*-score (Figure 1.1B). These analyses have detected the treatment potential of vitamin D on olanzapine-induced dyslipidemia. Although vitamin D was not among the drugs with the lowest ROR, which are presented to have preventing effects on dyslipidemia, vitamin D had the highest *Z*-score due to its sufficient number of reports, which guaranteed a better robustness for further investigation. However, vitamin D itself was associated with a slightly increased risk of dyslipidemia, according to FAERS data (ROR = 2.55, *Z*-score = 39.2). Therefore, further analyses to reinforce the present findings were performed with JMDC data.

Table 1.1 Association between atypical antipsychotics and the incidence of dyslipidemia in the FAERS database

Drug A	Dyslipidemia with drug A	Dyslipidemia without drug A	ROR	Z score
Olanzapine	2,716 / 59,859 (4.54%)	56,401 / 9,888,509 (0.57%)	8.29	105.3
Quetiapine	3,057 / 108,461 (2.82%)	56,060 / 9,839,907 (0.57%)	5.06	86.1
Ziprasidone	696 / 17,217 (4.04%)	58,421 / 9,931,151 (0.59%)	7.12	50.4
Risperidone	1,597 / 89,423 (1.79%)	57,520 / 9,858,945 (0.58%)	3.1	44.2
Aripiprazole	1,304 / 72,571 (1.80%)	57,813 / 9,875,797 (0.59%)	3.11	40.1
Clozapine	799 / 58,199 (1.37%)	58,318 / 9,890,169 (0.59%)	2.35	23.8

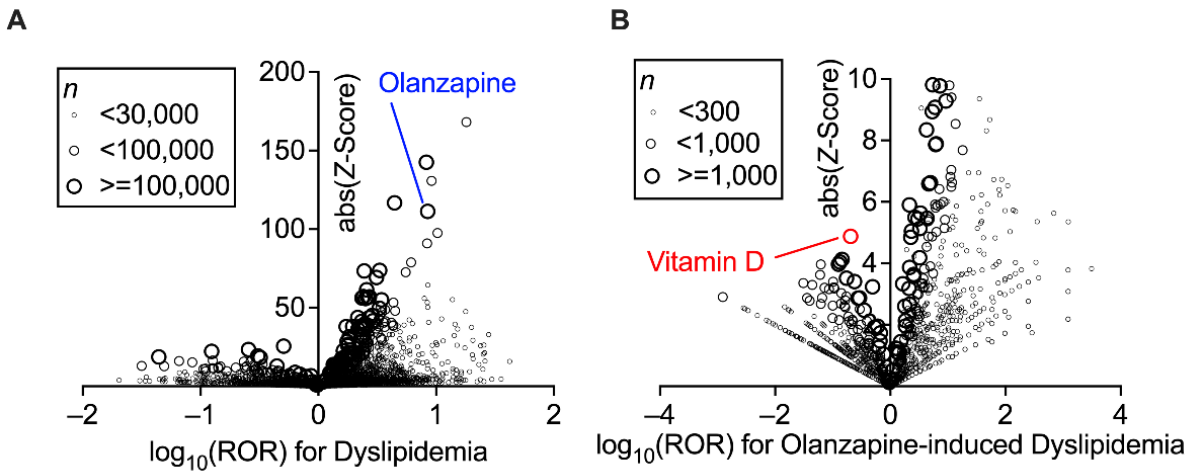

Figure 1.1 Increased incidence of dyslipidemia with the prescription of drugs and confounding effects of concomitant drugs on olanzapine-induced dyslipidemia in the FAERS data. Volcano plots for visualizing the reporting odds ratio (ROR, on a log scale) and its statistical significance (absolute Z score) are shown. Each circle indicates an individual drug, and the size of the circle reflects the number of patients taking the drug. (**A**) Strong and significant increases in the ROR for dyslipidemia were seen in patients using olanzapine. (**B**) Within the population taking olanzapine, confounding effects of concomitantly used drugs on the incidence of olanzapine-induced dyslipidemia were calculated thoroughly and plotted.

1.3.2. Analysis of JMDC Insurance Claims

To investigate whether the dyslipidemia incidence increase is caused by the clinical consequence of olanzapine treatment, the JMDC insurance claims were utilized for analysis. Blood test results in JMDC insurance claims from olanzapine-exposed patients with either vitamin D pre-treatment or without were extracted. Since vitamin D is predominantly taken by female elders, a balancing approach, propensity score matching, was applied to eliminate the background characteristics bias. After adjustments, no significant variance in baseline characteristics could be detected between patients with or without vitamin D pre-treatment (Table 1.2). The effect of vitamin D was investigated in balanced olanzapine-treated patients. In patients taking or not taking vitamin D, their blood triglyceride level is not influenced (Figure 1.2A); Nevertheless, the value of blood LDL cholesterol level was significantly increased in the olanzapine-only patients but not raised in olanzapine-vitamin D co-using patients (Figure 1.2B). Furthermore, blood HDL cholesterol level was significantly decreased in the olanzapine-only patients, while vitamin D supplements significantly counteracted this deteriorative change by even slightly increasing HDL cholesterol level (Figure 1.2C). These results indicated a rescuing effect of vitamin D on the impacts of olanzapine on blood lipid profiles.

Table 1.2 Population characteristics of the patients in the olanzapine cohort of the JMDC database; quantifiable data are given as means ± SEM

	With vitamin D	Without vitamin D	*P* value
Patients	20	20	NA
Elderly(≥ 65)	1 (5.0%)	1 (5.0%)	1
Female	18 (90.0%)	18 (90.0%)	1
Schizophrenia	4 (20.0%)	7 (35.0%)	0.48
Antipsychotics	6 (30.0%)	9 (45.0%)	0.51
Blood glucose (mg/dL)	91.9 ± 2.3	92.2 ± 3.1	0.94
HbA1c (%) (mg/dL)	5.49 ± 0.07	5.49 ± 0.12	0.97
Triglyceride (mg/dL)	97.6 ± 9.6	77.4 ± 12.0	0.2
LDL cholesterol (mg/dL)	120.7 ± 6.2	117.5 ± 5.1	0.69
HDL cholesterol (mg/dL)	68.1 ± 3.7	72.2 ± 3.4	0.42
BMI	20.3 ± 1.0	19.3 ± 0.6	0.38
Waist circumference (cm)	75.1 ± 2.5	71.2 ± 1.5	0.19

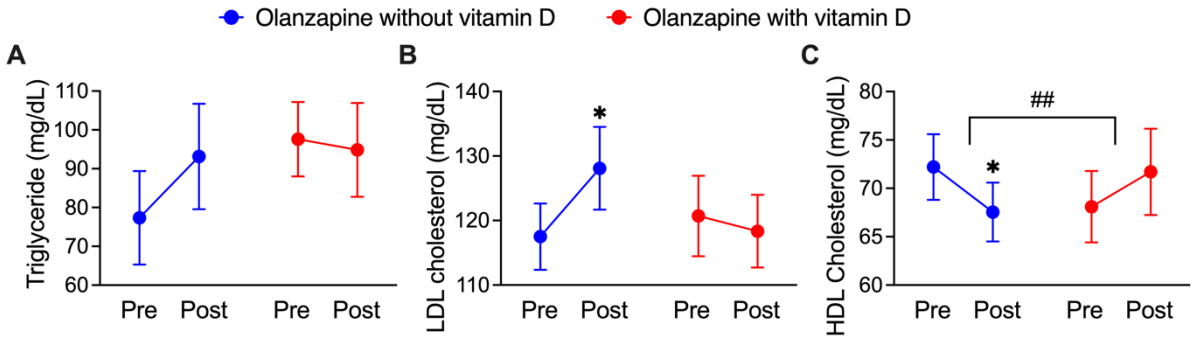

Figure 1.2 Deteriorative effects of olanzapine on blood lipid profiles and body weight maintenance and the rescuing effects of vitamin D co-treatment from the JMDC insurance claims. The data of pre-prescription and post-prescription periods were collected from the nearest test data before or after the olanzapine prescription within one year. Background characteristics were matched between groups ($n = 20$). **(A, B, C)** The annual blood test results from patients included in JMDC were extracted. (**A**) the triglycerides, (**B**) Low-density lipoprotein (LDL) cholesterol levels, and (**C**) High-density lipoprotein (HDL) cholesterol levels were expressed in units of mg/dL. Data are shown as means ± SEM. * $p < 0.05$; a paired *t*-test of the pre-prescription period against the post-prescription period. ## $p < 0.01$; an unpaired two-tailed *t*-test with Welch's correction between the group with and without vitamin D supplementation.

In addition to the blood lipid profiles, the effects of vitamin D on body mass, fat distribution, and blood glucose levels in olanzapine-treated patients were also investigated by analyzing related data in JMDC insurance claims. In the same groups providing blood test data, the body mass index (BMI), an indicator for a rough estimation of whether one is overweight (higher BMI representing a higher risk of overweighting), was significantly increased by olanzapine exposure, while the co-treatment with vitamin D diminished this trend (Figure 1.3A); The waist circumstances, an approximate indicator of body fat mass, also significantly increased in olanzapine-only treated patients, but not in patients co-treated with vitamin D. (Figure 1.3B); However, the blood glucose indicator, HbA1c, was not influenced in olanzapine-treated patients either with or without vitamin D exposures (Figure 1.3C). To summarize, these results indicate that the blood lipid profile deterioration, overweighting trend, and excessive fat accumulation are resultantly related to olanzapine treatment. At the same time, the combinative use of vitamin D has

beneficial effects in all these lipid metabolism indexes. Need to mention, the blood and body test data in JMDC insurance claims were collected from medical examinations provided by the Japan Health Insurance Association annually, which limited the preciseness of the detection of the onset and development of olanzapine-induced dyslipidemia. Thus, to improve this flaw, electronic medical records were obtained from the clinical data warehouse of NUSM, which is recorded in units of days, allowing the tracing of blood profile changes on a smaller but more precise scale.

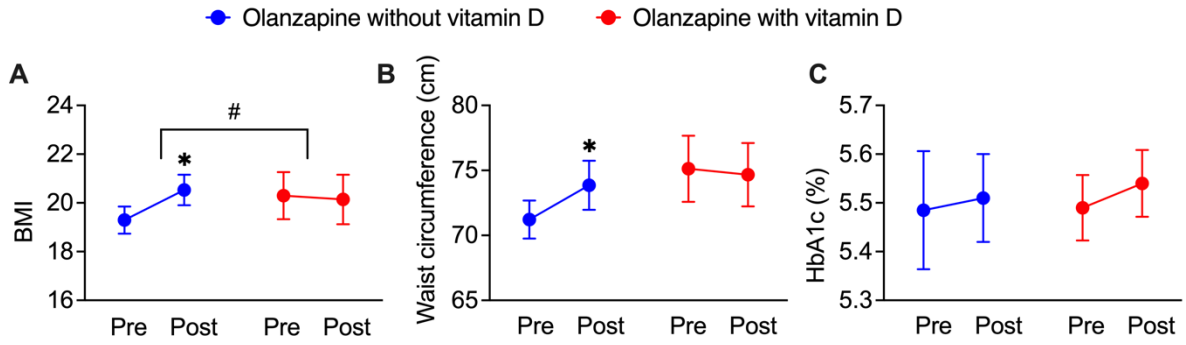

Figure 1.3 Deteriorative effects of olanzapine on body weight maintenance and the rescuing effects of vitamin D co-treatment from the JMDC insurance claims. The data of pre-prescription and post-prescription periods were collected from the nearest test data before or after the olanzapine prescription within one year. Background characteristics were matched between groups (n = 20). (**A, B**) The annual body weights, heights, and waist circumferences included in the JMDC were extracted. (**A**) Body mass index (BMI) is calculated from body weights and heights and (**B**) waist circumferences were obtained by direct measurement. (**C**) the level of HbA1c expressed in units of percentage of blood.

1.3.3. Analysis of Electronic Medical Records from the Clinical Data Warehouse of NUSM

Blood test data of patients exposed to olanzapine with either vitamin D or not were extracted from the electronic medical records, including triglycerides, LDL cholesterol, and HDL cholesterol values. No significant bias could be detected between the patients with vitamin D supplements or without (Table 1.3). By tracking the blood lipid profile changes continuously within one year after the first prescription of olanzapine, a significant increase in the blood triglycerides level was detected as early as 6 months after the start of treatment and kept increasing by 54mg/dL at the end of the year, while the co-treatment with vitamin D significantly diminished this trend and kept blood triglycerides level stable (Figure 1.4A); At the same time, significant increased blood LDL cholesterol level was detected in patients without vitamin D treatment not earlier than 12 months from their first prescription of olanzapine, but not in patients with vitamin D treatment. (Figure 1.4B) On the other hand, the HDL cholesterol level of blood from patients without D supplements reduced significantly after 6 months from their first olanzapine prescription (Figure 1.4C). Based on these observations, the beneficial effects of vitamin D treatments on olanzapine-induced dyslipidemia can also be detected within a shorter time, providing additional support to the findings from FAERS and JMDC databases.

Table 1.3 Population characteristics of the patients in the olanzapine cohort of the NUSM's clinical data warehouse; quantifiable data are given as means ± SEM

	With vitamin D	Without vitamin D	P value
Patients	406	23	NA
Age (years) mean	48.5 ± 0.90	57.1± 4.33	0.06
Female	249 (61.3%)	18 (78.3%)	0.12
Schizophrenia	110 (27.1%)	5 (21.7%)	0.81
Antipsychotics	225 (55.4%)	12 (52.2%)	0.83
Blood glucose (mg/dL)	107.6 ± 1.38	103.2 ± 4.67	0.38
HbA1c (%)	5.46 ± 0.04	5.55 ± 0.10	0.39
Triglyceride (mg/dL)	120.8 ± 4.63	135.2 ± 12.47	0.29
LDL cholesterol (mg/dL)	113.1 ± 2.85	125.5 ± 9.05	0.21
HDL cholesterol (mg/dL)	55.1 ± 1.04	52.9 ± 5.07	0.67
BMI	Not available	Not available	NA
Waist circumference (cm)	Not available	Not available	NA

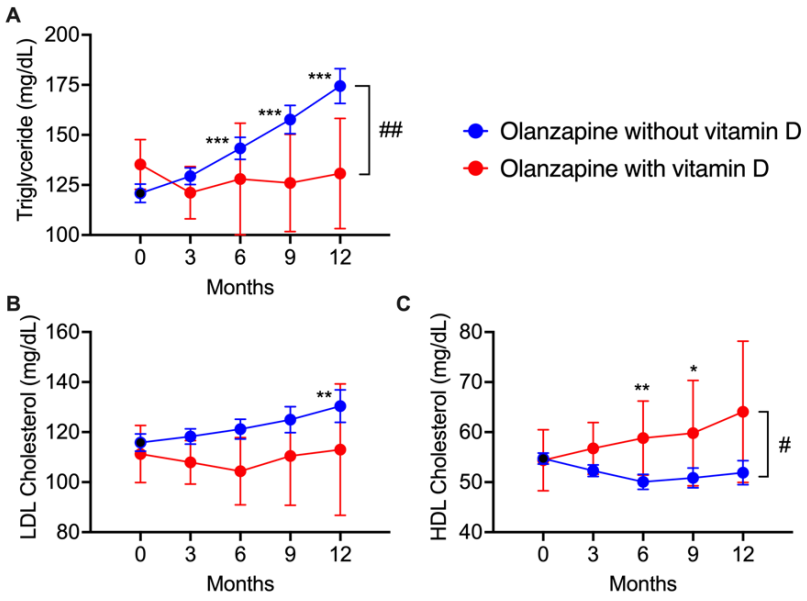

Figure 1.4 Deteriorative effects of olanzapine on blood lipid profiles and the rescuing effects of vitamin D co-treatment from electronic health records of the clinical data warehouse of Nihon University School of Medicine. Blood test results were extracted every three months after the first prescription of olanzapine. (**A**) the triglycerides (n = 406 in olanzapine without vitamin D group; n = 23 in olanzapine with vitamin D group), (**B**) the LDL cholesterol levels (n = 154 in olanzapine without vitamin D group; n = 11 in olanzapine with vitamin D group), and (**C**) the HDL cholesterol levels (n = 258 in olanzapine without vitamin D group; N= 16 in olanzapine with vitamin D group) were expressed in units of mg/dL. Data are shown as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. month 0 (baseline). # $p < 0.05$, ## $p < 0.01$. Data were analyzed using mixed-effects models.

1.4. Discussion

In the present study, the mitigative effects of vitamin D supplements on olanzapine-induced dyslipidemia in humans have been demonstrated for the first time. This finding has been firmly validated in three independent clinical databases with statistical methods and should be considered robust. In previous studies using the FAERS database, the drug repurposing-oriented approaches have been proven to be a reliable way to refine unexpected drug-drug interactive pairs with treating effects on adversary effects amelioration [20-22]. In the present study, olanzapine is one of the most dyslipidemia-inducing drugs, indicated by a high ROR value in more than tens of thousands of cases, providing a solid foundation for the exploration of potential rescuing drugs. Also, olanzapine is found to have a greater risk of inducing dyslipidemia than other atypical antipsychotics sharing similar molecular structures and action targets, such as clozapine and quetiapine, according to FAERS reports (Table 1.1). Furthermore, patients are generally more heavily dosed with clozapine ($C_{max} > 1.07$ μM) and quetiapine ($C_{max} \cong 0.21$ μM) than olanzapine ($C_{max} < 0.064$ μM) in clinical practice [26-28]. These two combined facts prioritize the screening on treating method of olanzapine-induced dyslipidemia from other atypical antipsychotics. On the other hand, vitamin D treatment is also recognized as a dyslipidemia risk factor aside from olanzapine, which is likely caused by the confounding in identifying the causes and results, the fact of which can be explained by an example: The use of a drug treating specific disease is surely positively related to the incidence of this disease as patients with this disease need to take this drug for treatment. This confusion is due to the lack of information about the prescription date to help define the sequence of drug exposure and disease onset. Anyway, this problem was further solved with cross-validations with health databases, including records on the prescription date in the present study.

The hypothesis developed from FAERS data analysis was further testified in a retrospective cohort analysis based on JMDC insurance claims. As a supplement to FAERS data, JMDC insurance claims provided blood test results of patients under 75 annually. Therefore, JMDC data allows the analysis of the casual relationship between olanzapine exposure with blood lipid profile changes. In the present study, olanzapine-induced dyslipidemia and weight gaining have been revealed in patients without vitamin D. However, the background characteristics of the patients are strongly biased in gender (primarily women), which is because that vitamin D is predominantly prescribed to women in the case that they are more susceptible to osteoporosis [29]. Nevertheless, in previous clinical reports, olanzapine has been reported to be able to induce LDL cholesterol level and triglycerides levels in both adolescents and young adults, regardless of gender variance, indicating that the analysis results from JMDC claims could still have high consistency with clinical observations [14, 30]. On the other hand, the present study found that patients supplementing with vitamin D during the olanzapine treatment did not exhibit increased LDL cholesterol levels, decreased HDL cholesterol levels, increased BMI, nor increased waist circumferences. This study, for the first time, exhibits the clinical significance of vitamin D in preventing olanzapine-induced dyslipidemia, as so far, only one trial has shown that vitamin D has beneficial in maintaining waist circumference in olanzapine-treated patients [31].

Although the JMDC database is already efficient in solidifying the discovery of olanzapine-vitamin D drug interaction, it is still unable to precisely identify the onset pattern of olanzapine-induced dyslipidemia due to the long intervals between every collecting of data. Improvements were made by introducing electronic medical records from the clinical data warehouse of NUSM, the electronic records of which are updated daily, allowing a flexible set of time scales for analysis. By the blood test results

aggregated every three months after olanzapine treatment started, the triglycerides and LDL cholesterol levels were found to increase monotonically as the exposure time accumulated, and the significance of triglycerides level changes appeared ahead of that of either LDL cholesterol level changes or HDL cholesterol level changes. These details can only be able to find in the NUSM database but not in JMDC or FAERS databases, suggesting an advantage in analysis. Unlike the analyzed results from JMDC database on triglycerides, by which the triglycerides levels were passively influenced, the patients in NUSM displayed a strong trend in developing hypertriglyceridemia. This inconsistency between results from different databases can be interpreted by the variance in population background characteristics, as patients included in electronic records from the clinical data warehouse of NUSM are more balanced in gender distributions (female ratio = 61.3%) than those from JMDC insurance claims (female ratio = 61.3%). Higher estrogen level in females is generally recognized as the reason for their better triglyceride control than males, which may account for the differences in triglyceride level changes [32]. Vitamin D treatment counteracted these deteriorative changes by maintaining the blood lipid profile at stable levels throughout the observation window, consistent with the outcomes from JMDC claims. By joining all these three outcomes based on different data sources, a causal relationship between dyslipidemia and olanzapine treatment was firmly established, and the suppressive effects of vitamin D supplements on olanzapine-induced dyslipidemia were also confirmed.

Chapter 2. Experimental validation for the effect of vitamin D on olanzapine-induced dyslipidemia

2.1. Introduction

Olanzapine was originally approved in 1996 as an improved analog of clozapine with less hematological risk [33]. In primary *In vitro* studies, olanzapine was reported to have a high binding affinity with dopamine receptors D1, D2, and D4, together with serotonin receptors 5HT2A, 5HT2C, and 5HT3m [2, 34]. Further investigations by *in vivo* models demonstrated behavior changes related to dopamine receptors blockade and serotonin receptors blockade [35, 36], clarifying the mechanism of the treating effects of olanzapine. Most of the adverse effects caused by olanzapine are associated with its primary targets, such as extra-pyramidal side effects and endocrine side effects [37]. However, the primary targets of olanzapine can hardly be found in lipid metabolism-related tissues or organs in the human body, such as the liver, adipose tissue, and muscle tissue, according to the human proteome database Human Atlas [38]. So far until now, no off-target effects have been reported in olanzapine, making the mechanism of olanzapine-dyslipidemia an enigma. In recently developed drug-induced disease models in rodents on olanzapine, pathological changes in the liver have been highlighted as possible causes of dyslipidemia and obesity. Non-alcoholic fatty liver diseases have been reported by several previous studies, implicating the direct hepatotoxicity of olanzapine as the primary mechanism [39-42]. Also, in *in vitro* explorations, olanzapine has been reported to induce lipid biosynthesis gene expression in hepatocytes and adipocytes and cause lipid accumulation [43, 44]. The mechanism of these observations has been assumed as olanzapine inhibits cholesterol esterification, further triggering cholesterol-thirsty-induced lipid biosynthesis [45]. So far, none of these results have been solidified. Therefore, further validation of the

direct association of olanzapine with lipid dysregulation is required in the experimental part of this study.

Vitamin D is known as an essential nutrient that can be either absorbed from foods or produced by biosynthesis from cholesterol [46]. Traditionally, vitamin D is considered to be a primary regulator of calcium absorption in the gastrointestinal tract and a supporter of bone formation [47]. Its role in preventing dyslipidemia has not yet been focused on up to now. Clinical studies have established a common understanding that vitamin D deficiency is positively related to the incidence of obesity, dyslipidemia, and cardiovascular diseases [48-50]. However, the casual relationship still needs to be clarified. A hypothesis has been raised as: vitamin D tends to translocate to adipose tissue due to its lipophilicity, and excessive adipose tissue will keep too much vitamin D from entering metabolism and cause available vitamin D amounts to drop [51]. From this assumption, vitamin D deficiency seems to be resultantly related to lipid metabolism disorders. However, a large number of clinical trials demonstrated the preventing effects of vitamin D on dyslipidemia, suggesting another unknown mechanism [52, 53]. Currently, a limited number of studies focus on the mechanism behind these observations because the liver, the cholesterol metabolism center in the body, expressed no VDR and acted passively toward vitamin D stimulations [54]. This part of the present study aims to clarify the possible mechanism of the preventing effects of vitamin D on dyslipidemia by testing its actions in possible lipid metabolism-related tissues.

2.2. Methods

2.1.1. Animals

All animal experiments were approved by the Kyoto University Animal Research Committee in accordance with the ethical guidelines of the committee. All experiments were designed to minimize the use of animals and the number of

experiments. Male and female C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan) and housed with a constant ambient temperature (24 ± 1°C) and humidity (55% ± 10%) on a 12h/12h light/dark cycle Mice were fed with water and chowed diet *ad libitum*.

2.1.2. Reagents and Treatments (*in vivo*)

Olanzapine was purchased from Tokyo Chemical Industry (Tokyo, Japan). The medium-fat diet (containing 1.37 IU VD_3/g) and the VD_3-supplemented medium-fat diet (containing 200 IU VD_3/g) were purchased from Oriental Yeast (Tokyo, Japan). Olanzapine was dissolved in water with additional 0.5% carboxymethyl cellulose before use. VD_3 is administrated orally as a chow supplement; the intaking dose may vary slightly among mice. Mice were randomized into two groups, fed with a medium-fat diet or a VD_3-supplemented medium-fat diet for one week. Mice from each group were further randomized to be treated with olanzapine (10 mg/kg, orally administered) or vehicle (0.5% carboxymethyl cellulose solution) for another five days. One day after the last dose of olanzapine, mice were anesthetized and dissected for blood collected by cardiac puncture. Mice serum was isolated from blood samples by centrifugation. Respectively, the total cholesterol level in serum was measured with LabAssay Cholesterol kit (Wako, Osaka, Japan), while the measurement of serum LDL cholesterol, HDL cholesterol, and triglyceride levels measurement were assigned to Nagahama Lifescience (ORIENTAL YEAST CO., Ltd, Shiga, Japan).

2.1.3. Cell Culture

2.1.3.1. Mouse hepatocyte primary culture

Primary mouse hepatocytes were prepared as previously described with modifications [55, 56]. Livers from 6-8 weeks old male mice were perfused with

HBSS without calcium, magnesium, and phenol red, supplemented with 0.5 mM of EDTA and 25 mM of HEPES and followed by perfusion with digestive enzyme mix (collagenase, type I (Worthington, NJ, USA) 0.15 mg/mL; collagenase, type II (Worthington, NJ, USA) 0.15 mg/mL; Dispase, type II (Gibco, MA, USA) 0.15 mg/mL) solution, dissolved in standard HBSS with phenol red, supplemented with 25 mM of HEPES. Hepatocytes were released into high-glucose Dulbecco′s modified eagle′s medium (DMEM; D5796; Sigma-Aldrich, MO, USA) medium from digested livers, filtered through 70 μm cell strainers (Corning, NY, USA) and further purified with 45% Percoll® cushion by density gradient centrifugation. Purified cells were re-suspended in William's E medium (A1217601; Gibco) supplemented with 5% FBS Sigma-Aldrich), 1 μM of dexamethasone (Nacalai Tesque, Kyoto, Japan), 1% Penicillin-Streptomycin Mixed Solution (P/S; Nacalai Tesque), 5 μg/mL of human recombinant insulin (Sigma-Aldrich), 2 mM of GlutaMAX™ supplement (Sigma-Aldrich), and 15 mM of HEPES. Cells were plated onto dishes coated with 0.1 mg /mL of collagen type I (Nippi, Tokyo, Japan) and incubated at 37°C in a humidified chamber containing 5% CO2 for 3h. After attaching to the surface, the medium was refreshed with serum-free William's E supplemented with 1 μM of dexamethasone, 0.5% P/S, 1% ITS+ premix (Corning), 2 mM of GlutaMAX™ supplement, and 15 mM of HEPES. Cells were utilized for treatment within a maximum extent of 48 h.

2.1.3.2. Mouse primary adipocyte culture

Primary mouse adipocytes were prepared as previously described with modifications [57, 58]. Subcutaneous adipose tissues were collected from neonatal mice pups and digested with 0.1 mg/mL of collagenase, type II, and 0.1 mg/mL dispase, type II, dissolved in standard HBSS. Digested tissues were homogenized

by pipetting, filtered through 70 μm cell strainers, and then resuspended in high-glucose DMEM supplemented with 20% FBS, 1% P/S, and 10 mM of HEPES. Collected preadipocytes were plated onto dishes coated with 0.1% gelatin (Nacalai Tesque) in distilled water. The cells were flushed with HBSS twice and refreshed with the same medium. After the cells reached 90% confluency, cells were transferred into high-glucose DMEM supplemented with 10% FBS, 1% P/S, 10 mM of HEPES, 170 nM of human recombinant insulin, 1 μM of dexamethasone, 0.5 mM of 3-isobutyl-1-methylxanthine, 1 nM of triiodothyronine, and 10 nM of hydrocortisone for differentiation to adipocytes. After 48 h, the cells were further transferred to high-glucose DMEM supplemented with 10% FBS, 1% P/S, 10 mM of HEPES, 170 nM of human recombinant insulin, and 1 nM of triiodothyronine. After 5-day maintaining, the cells were ready for treatment.

2.1.3.3. C2C12 cell line culture

Mouse C2C12 cells were prepared as previously described with modifications [20]. Cells were obtained from Prof. H. Takeshima (Kyoto University, Graduate School of Pharmaceutical Sciences, Kyoto, Japan). Cells were thawed in a water bath at 37°C and plated with high-glucose DMEM supplemented with 10% FBS, 1% P/S, and 10 mM of HEPES. After reaching 70% confluency, cells were digested with 0.25% trypsin solution (Nacalai Tesque) and passaged in the same medium. When cells reached 90% confluency, the medium was changed to high-glucose DMEM supplemented with 2% horse serum (Sigma-Aldrich), 1% P/S, and 10 mM HEPES for differentiation. The medium was refreshed 3 days after the changes, and cells were ready on day 5.

2.1.4. Reagents and Treatments (*in vitro*)

25(OH)D_3 was purchased from Selleckchem (TX, USA), and 1,25$(OH)_2D_3$ was purchased from Cayman (MI, USA). ZK159222 was purchased from Cayman. All reagents were reconstructed and preserved in DMSO as a vehicle at -80 °C, and thus DMSO was added as a blank reference in control groups. Primary mouse hepatocytes were treated in the maintaining medium. Primary mouse adipocytes and mouse C2C12 cells were treated in Advanced DMEM/F12 medium (12634010, Gibco) supplemented with 1% P/S, 2 mM of GlutaMAX™ supplement, and 10 mM of HEPES.

2.1.5. Gene expression quantification

2.1.5.1. RNA-seq analysis

Total RNA was isolated from cultured cells, following standard protocols provided by Isogen reagents (Nippon Gene, Tokyo, Japan). In RNA-Seq analysis, poly$(A)^+$ RNA was selected from total RNA and sequenced using DNBseq (BGI, Shenzhen, China). Total reads were filtered by SOAPnuke [59]. Clean reads were mapped to the mouse reference genome GRCm38.p6 with HISAT, version 2.0.4 and Bowtie, version 2.2.5 in parallel [60, 61]. Gene expression was calculated using RSEM, version 1.2.8 [62]. The cholesterol biosynthetic process-related gene set was obtained from MGI (http://www.informatics.jax.org/vocab/gene_ontology/GO:0006695) [63]. All the genes with reads (transcript per million > 1) from three types of culture cells (hepatocytes, adipocytes, and C2C12 cells) were mapped to this gene set to generate a list of the cell-specific cholesterol biosynthetic process-related genes. The detection of differentially expressed genes between vehicle and olanzapine, olanzapine and olanzapine-25(OH)D_3 co-treated, and olanzapine and olanzapine-

1,25$(OH)_2D_3$ co-treated treated cells was performed based PossionDis [64]. Genes that changed with fold-change (FC) ≥2 and false discovery rate (FDR) ≤0.05 were recognized as differently expressed. The analyzed results were visualized with volcano plots drawn using Prism 9.4.1 (GraphPad Software, CA, USA), with -LOG(FDR) in the y direction and LOG2(FC) in the x direction. The original sequence datasets were deposited to the NCBI sequence read archive under accession number: GSE221683.

2.1.5.2. qRT-PCR experiments

Total RNA was isolated from cultured cells, following standard protocols provided by Isogen reagents. Extracted RNA was translated to cDNA using ReverTra Ace (Toyobo, Osaka, Japan) and subjected to StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA, United States) with Thunderbird SYBR qPCR Mix (Toyobo). The amplification process was set as follows: 10 min at 95°C followed by 40 cycles that looped from 15 s at 95°C to 60 s at 60°C. The oligonucleotide primers were purchased from Sigma-Aldrich, and the sequences are as follows (written as gene name: 5'-forward-3', 5'-reverse-3'): Mus *Rplp0*: GCT TCG TGT TCA CCA AGG AGG A, GTC CTA GAC CAG TGT TCT GAG G; Mus *Hmgcr*: GCT CGT CTA CAG AAA CTC CAC G, GCT TCA GCA GTG CTT TCT CCG T; Mus *Insig2*: GCC CAT CCA GAA CCT CTG AC, AGA CGG GGC AAA AGG ACT TC. The expression levels of each mRNA was normalized to that of *Rplp0* mRNA.

2.1.6. Western blotting

Western blotting was performed as described previously with modifications [65]. Cells were lysed in RIPA buffer (Nacarai Tesque), diluted to 1 μg/mL with 1% SDS water solution, and then buffered with NuPAGE® 4× LDS sample buffer (Life

Technologies, CA, USA). Samples were loaded onto 10% SDS-polyacrylamide gel and blotted onto ClearTrans® PVDF Membrane (Wako). After being blocked with Blocking One (Nacarai Tesque), the membranes were split into two pieces to blot the target protein, VDR, and endo-reference protein, GAPDH, simultaneously. The membranes were incubated overnight at 4°C with anti-VDR antibody (1:500 dilution, 12550, Cell Signaling Technology, MA, USA) and anti-GAPDH (1:50,000 dilution, 32233, Santa Cruz, CA, USA) in Tris-buffered saline supplemented with 0.1% Tween 20 (TBS-T) and 10% Blocking One. After washing with TBS-T, membranes were respectively incubated with peroxidase-conjugated donkey anti-rabbit IgG (1:5000 dilution, NA934V, GE Healthcare, IL, USA) and Peroxidase AffiniPure Goat Anti-Mouse IgG (1:5000 dilution, 115-035-003, Jackson ImmunoResearch, PA, USA) for 2 h at RT. Specific bands were detected by Immobilon Western Chemiluminescent HRP substrate (Millipore) and visualized using the EZCapture MG (ATTO, Tokyo, Japan). The expression levels of VDR were normalized to that of the GAPDH level.

2.1.7. Statistics

Statistical analysis of the animal experiments was performed with GraphPad Prism 9.3.1, and the data were expressed in the mean ± standard error of mean (SEM). The blood profile data were analyzed by two–way analysis of variance (ANOVA) with *post hoc* Tukey's Multiple Comparison Test. The qRT-PCR revalidations in C2C12 cell line on RNA-Seq resultant data were analyzed by two–way analysis of variance (ANOVA). The dose-depend changes of specific genes responding to $25(OH)D_3$, $1,25(OH)_2D_3$, and ZK159222 in the C2C212 cell line were analyzed by one–way analysis of variance (ANOVA). The western blotting results

of VDR cell-specific enrichment were analyzed with Student's t-test. All reported *P*-values of less than 0.05 were considered to indicate statistical significance.

2.3. Results

2.3.1. Effects of vitamin D on an olanzapine-induced dyslipidemia mouse model

Previously established olanzapine-induced dyslipidemia models are generally based on chronic treatments lasting at least 4 weeks, causing severe liver pathology as an outcome [39-42]. In the present study, in order to study the primary stage of dyslipidemia development, the treatment was limited to 5 days. To mimic the calorie intake of human patients, we supplemented fructose to the drinking water of mice at a final concentration of 30% during the 1 week of acclimatization and continued in the following runs of treatment [66]. The treatment time course is illustrated by Figure 2.1A. As for results to show, neither a scheduled administration of olanzapine (10 mg/kg, orally administrated by gavage, daily for 5 days) nor vitamin D supplements (administrated via a VD_3-supplemented diet containing 200 IU VD_3/g) caused significant changes in blood triglyceride level (Figure 2.1B) and total cholesterol level (Figure 2.1C). On the other hand, a significant increase in the blood LDL cholesterol level and attenuating effects of vitamin D supplements could be detected (Figure 2.1D). Also, the simultaneous reduction of HDL cholesterol caused by olanzapine exposure was attenuated by vitamin D supplements (Figure 2.1E). These findings suggested that a short-term administration of olanzapine is sufficient to induce dyslipidemia, characterized by the broken balance of blood cholesterol distribution between LDL and HDL, without influencing the blood triglyceride and the total cholesterol level. On the other hand, the supplementation of vitamin D in the form of VD_3 diminished these impacts.

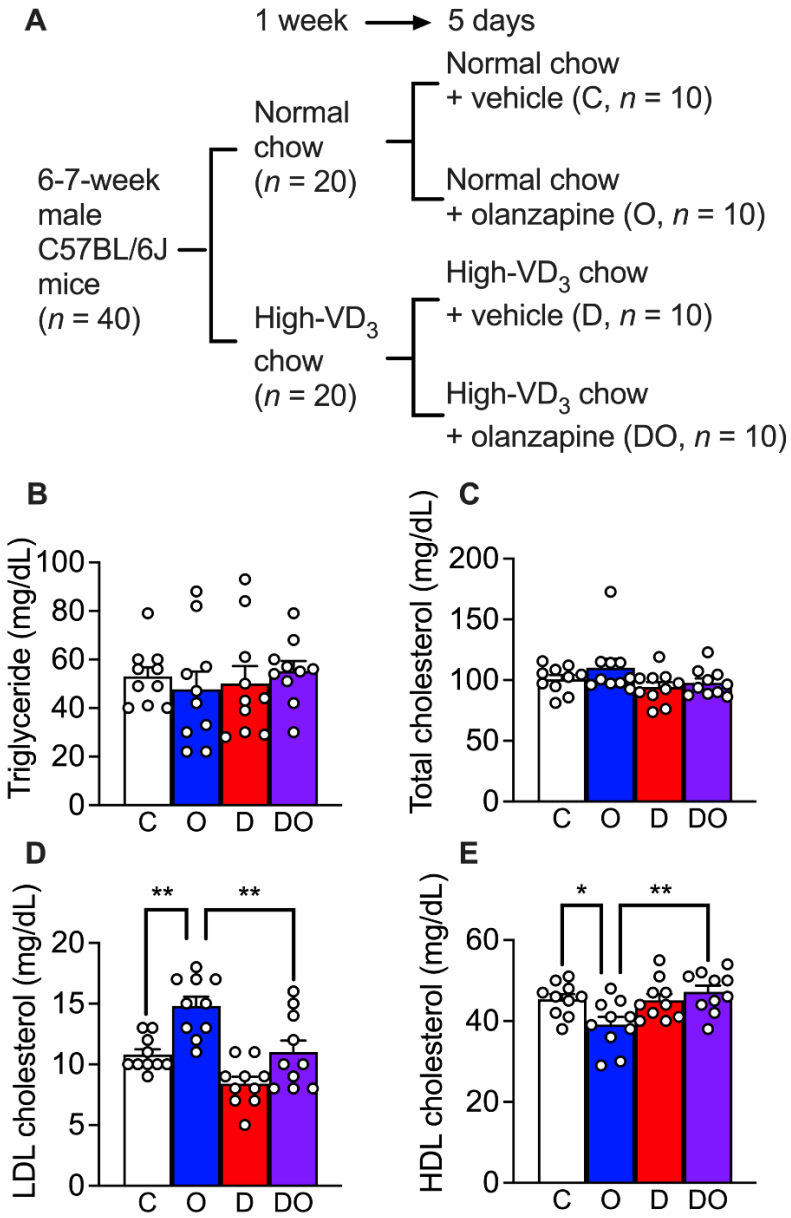

Figure 2.1 Olanzapine treatment caused dyslipidemia in a rodent model while vitamin D3 supplements attenuated the worsening of the condition. (A) C57BL/6J mice were fed for one week with the medium-fat diet (containing 1.37 IU of cholecalciferol/g of chow) or cholecalciferol-supplemented medium-fat diet (containing 200 IU of cholecalciferol/g of chow). Each group of mice were divided into two groups and orally given 10 mg/kg/day of olanzapine or vehicle for 5 days; resulting 4 groups of mice (each n = 10) received normal chow and vehicle as control (C), normal chow and olanzapine (O), vitamin D3-supplemented chow and vehicle (D), and vitamin D3-supplemented chow and olanzapine (DO). Blood samples were collected from these mice one day after the last dose of olanzapine without fasting. (B) the triglycerides, (C) total cholesterol levels, (D) LDL cholesterol levels, and (E) HDL cholesterol levels were measured. Data are shown as means ± SEM. The multiple comparisons of olanzapine and cholecalciferol influences were compared by two-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$.

2.3.2. Exploration of molecular mechanisms of the treating effects of vitamin D on olanzapine-induced dyslipidemia with RNA-seq

To investigate the molecular mechanisms underlying cholesterol dyshomeostasis in blood under the short-term olanzapine and also the rescuing effects from VD_3 supplements, the gene expression changes in cholesterol metabolism-related cells were further investigated with RNA-seq. In the present study, the purified cultures of mouse hepatocytes, adipocytes, and fully differentiated C2C12 cells (myotubes) were utilized instead of liver, adipose, and skeletal muscle tissues as these tissues are general mixtures of various cells with complicated origins and functions, which may blur the direct influence from olanzapine and vitamin D. Moreover, for a comprehensive study, both of the main form of vitamin D under physiological environment, the $25(OH)D_3$ and $1,25(OH)_2D_3$, were utilized for the *in vitro* treatment. All three types of cells were treated under the same condition, a 24 h pre-exposure to $25(OH)D_3$ (10 μM, the relatively inactive form of VD_3) or $1,25(OH)_2D_3$ (0.1 μM, the active form of VD_3) and another 12 h co-treatment with olanzapine (1 μM), separately. As only the cholesterol homeostasis was broken in the blood according to the *in vivo* model from the present study, only the cholesterol biosynthesis-related genes obtained from the MGI database were selected for analysis [63]. As for results to show, the expression of a set of genes was significantly changed in olanzapine-$25(OH)D_3$ co-treated C2C12 cells and slightly changed in olanzapine-$1,25(OH)_2D_3$ co-treated C2C12 cells. On the other hand, olanzapine displayed passive effects on all three types of cells (Figure 2.2A). For further exploration, the regulative relationship within this set of genes was illustrated by a scheme (Figure 2.2B). From the scheme, the *Insig2* gene, a suppressive regulator of cholesterol biosynthesis, was upregulated by vitamin D

metabolites, while, at the same time, the genes participating in the mevalonate pathway, like *Hmgcs1*, *Hmgcr*, *Fdps*, *Fdft1*, *Lss*, *Cyp51*, *Nsdhl*, and *Dhcr24* were significantly downregulated [67, 68].

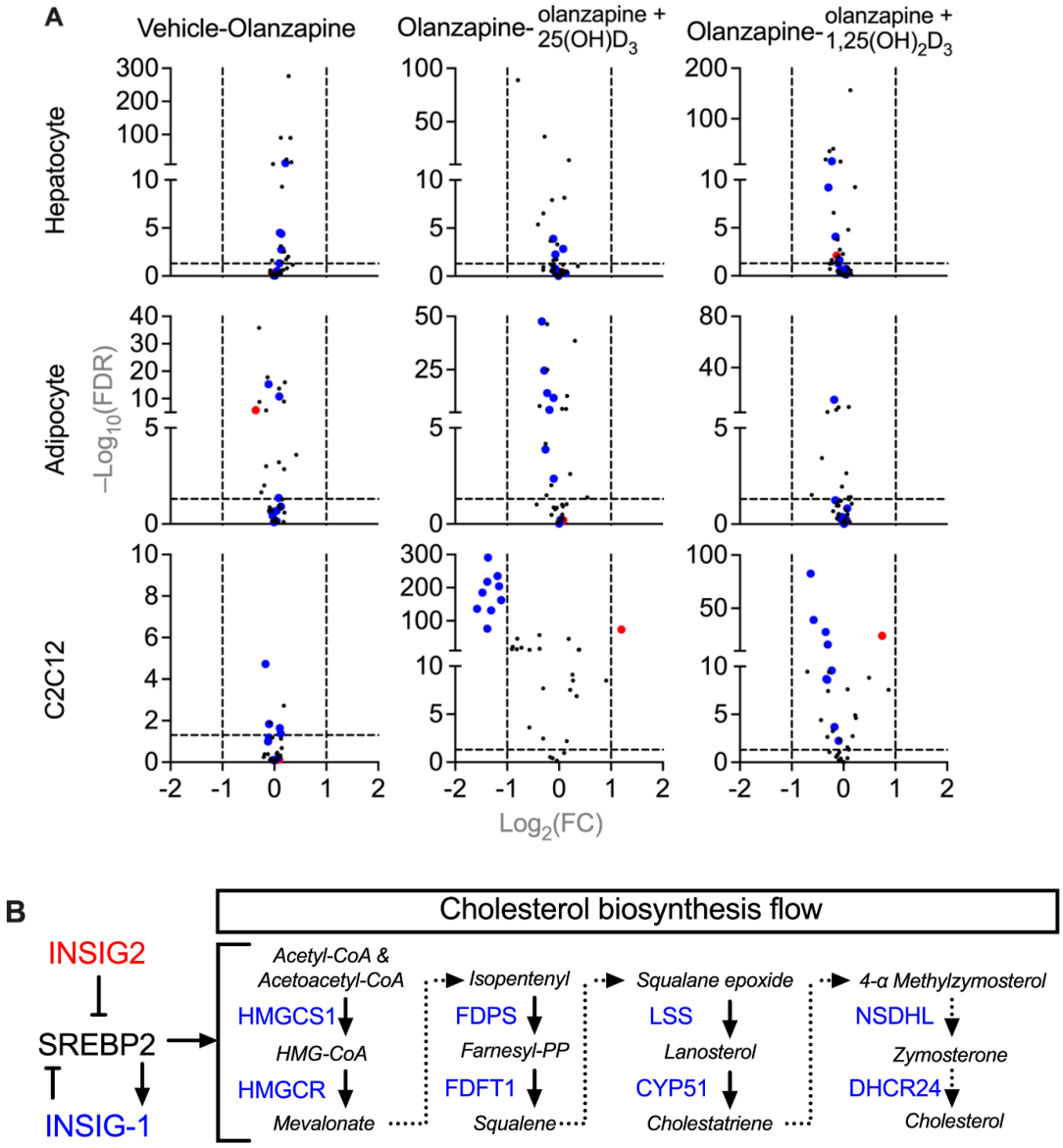

Figure 2.2 Volcano plots of gene expression in the cultured mice cells treated with vehicle, olanzapine, or olanzapine co-treated with 25(OH)D_3 or 1,25(OH)$_2D_3$. The changing ranges were expressed as Log2-transformed fold-change (FC), and the significance was expressed as -Log10-transformed false discovery rate (FDR) (n = 1). (**A**) Demonstrations of the cholesterol biosynthesis-related gene changes under olanzapine treatment and 25(OH)D_3 or 1,25(OH)$_2D_3$-olanzapine co-treatment. The genes significantly downregulated in the olanzapine-25(OH)D_3 co-treated C2C12 cells are tagged with blue dots, and the genes upregulated are tagged with red dots in all the data sets. (**B**) A schematic diagram illustrating the functions of downregulated genes (blue) and upregulated gene (red) in the process of cholesterol biosynthesis. Genes plotted with FC absolute value >2 and FDR value >0.05 were considered to be significantly changed by treatment.

2.3.3. The investigation of the molecular mechanism of the 1,25(OH)$_2$D$_3$-induced suppression of cholesterol synthesis

For further validation of the RNA-seq analysis results, an enlarged batch of testing was performed on C2C12 cells, which displayed the highest sensitivity to 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$, with qRT-PCR. Hmgcr was selected as a representative gene of the cholesterol biosynthesis process as it is recognized as the master pace-limiting enzyme of the whole flow [69]. Results by qRT-PCR showed that using the same treatment as the RNA-seq experiment, the suppression of *Hmgcr* expression (Figure 2.3A) and inducing of *Insig2* expression (Figure 2.3B) by 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ could be confirmed. A previous report claimed that 25(OH)D$_3$ but not 1,25(OH)$_2$D$_3$ is able to reduce cholesterol biosynthesis without VDR action [70]. However, further validations by changing the treating concentration of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ found that the expression changes of *Hmgcr* (Figure 2.3C) and *Insig2* (Figure 2.3D) followed a dose-depending manner in 25(OH)D$_3$-treated cells. In parallel, the expression changes of *Hmgcr* (Figure 2.3E) and *Insig2* (Figure 2.3F) followed a dose-depending manner in 1,25(OH)$_2$D$_3$-treated cells, with approximately 5.8 times higher sensitivity (IC_{50} of calcifediol on Hmgcr: 0.68 μM; IC_{50} of calcitriol on Hmgcr: 0.12 μM;). As 1,25(OH)$_2$D$_3$ is the bioactive form of vitamin D which acts only through VDR [46], the role of VDR in controlling cholesterol biosynthesis in this model needs further investigation.

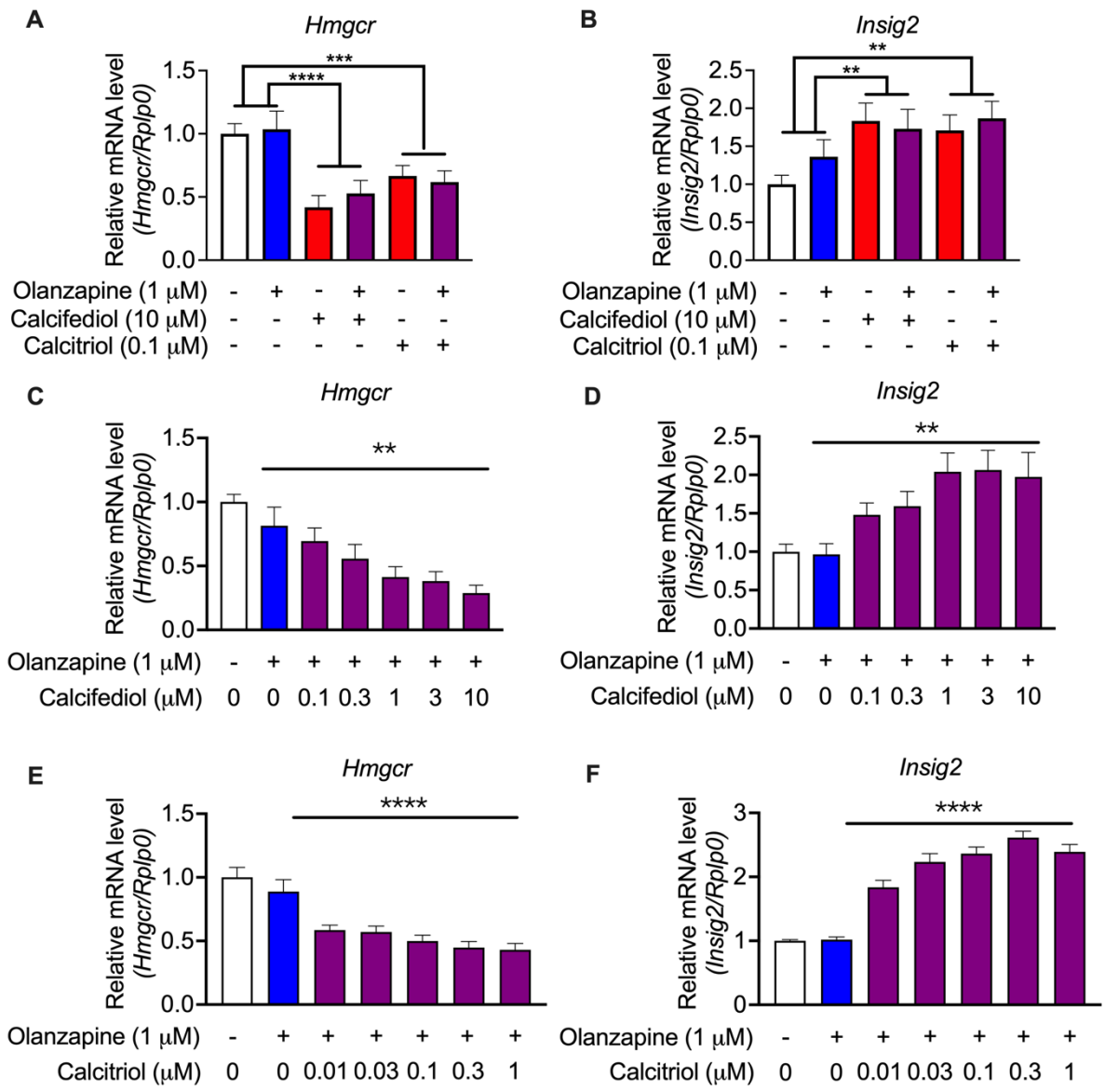

Figure 2.3 Validation by quantitative RT-PCR results showing the expression of cholesterol biosynthesis-associated genes in the cultured C2C12 cell line. (**A**) *Hmgcr* and (**B**) *Insig2* were validated in the same treating condition as that in the RNA-seq experiments (n = 12). Results were normalized to *Rplp0* (**C**) *Hmgcr* and (**D**) *Insig2* were validated in 25(OH)D_3 with gradient-changed concentrations increasing from 0 μM to 10 μM (n = 8–9). (**E**) *Hmgcr* and (**F**) *Insig2* were validated in 1,25(OH)$_2D_3$ with gradient-changed concentrations increasing from 0.01 μM to 1 μM. Data are shown as means ± SEM (n = 9). ** $p < 0.01$, **** $p < 0.0001$. The comparisons of olanzapine and vitamin D influences were compared by two-way analysis of variance (ANOVA). The validation of the dose-dependent effects of 25(OH)D_3 and 1,25(OH)$_2D_3$ were compared by one-way ANOVA.

2.3.4. The investigation on the role of VDR in the controlling cholesterol biosynthesis

To validate the action of VDR in this process, western blotting of the VDR protein levels in the above-mentioned three types of cells was performed. The results show that VDR is highly expressed in C2C12 cells, much higher than in adipocytes and hepatocytes (Figure 2.4A). The varied tissue-specific enrichment of VDR indicated the induction of *Insig2* and the suppression of *Hmgcr* expression is probably mediated by VDR activity. Further, a partial agonist of VDR, ZK159222, is applied to validate the action of the VDR [71]. ZK159222, like $25(OH)_2D_3$ and $1,25(OH)_2D_3$, suppressed Hmgcr expression (Figure 2.4B) and induced Insig2 expression (Figure 2.4C) in a dose-depend manner, further solidifying the essential role of VDR.

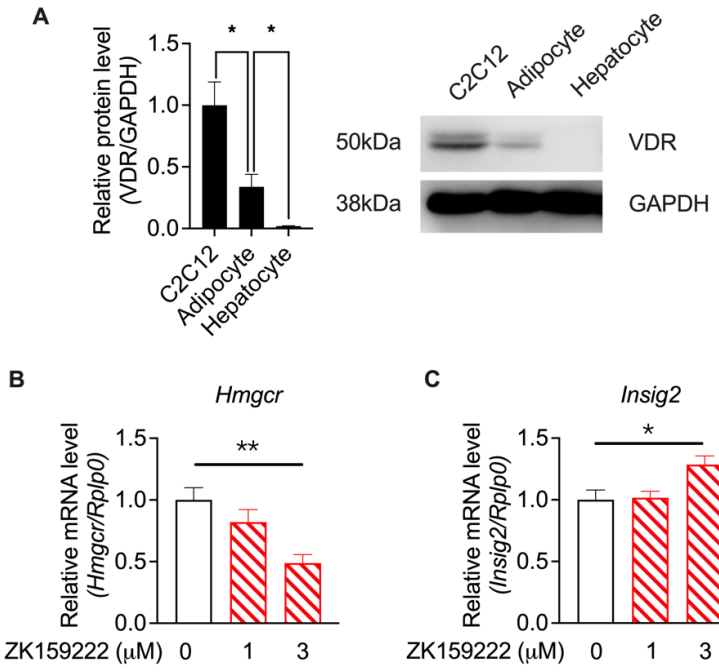

Figure 2.4 Validation by quantitative RT-PCR results showing $1,25(OH)_2D_3$ regulating the cholesterol biosynthesis-associated genes mediated by VDR in the C2C12 cell line. (**A**) The results of vitamin D receptor (VDR) protein levels in C2C12 cells, adipocytes, and hepatocytes, normalized to GAPDH ($n = 3$). (**B**) *Hmgcr* and (**C**) *Insig2* were validated in ZK159222 with gradient-changed concentrations increasing from 0 μM to 3 μM ($n = 6$). Data are shown as means ± SEM. * $p < 0.05$, ** $p < 0.01$. The validation of the dose-dependent effects of ZK150222 was compared by one-way analysis of variance (ANOVA).

2.4. Discussion

In the present study, the hypothesis of the protective effect of vitamin D was validated by the blood profiles of mouse models of olanzapine-induced dyslipidemia. Furthermore, the possible mechanism was further investigated in cells that were metabolically-realted to cholesterol. In the present study, the disruption of blood lipid balance in mouse blood can be detected as soon as 5 days after olanzapine treatment, the results of which largely differed from the previous reports, as it took at least 4 weeks for the symptoms to occur [39-42]. This inconsistency may be attributed to different goal settings, as the above-mentioned studies generally used visible lipid droplets in the liver as the outcome of olanzapine influence, while the present study only took the occurrence of cholesterol homeostasis breaking as the endpoint of observation. However, olanzapine-induced dyslipidemia is rarely accompanied by liver pathologies, according to clinical observations [72]. The non-acholic fatty liver was exclusively driven by extreme weight gain during long-term treatment in patients, as reported by another clinical study [73]. Nevertheless, a recent clinical trial found that the raised appetite is essential for patients to develop olanzapine-induced dyslipidemia [74], the result of which can be explained by an earlier case report: enhanced food reward circuitry has been detected in olanzapine-treated patients [75]. This phenotype has been established in rodent models and was attributed to the antagonistic effects on histamine H_1 receptors in the central nervous system by olanzapine [76, 77]. As a conclusion, the hepatotoxicity is not casually related to olanzapine-induced dyslipidemia but probably contributed by the metabolic stress raised by olanzapine-induced appetite increase. However, the role of vitamin D in lipid metabolism remains controversial. Several studies have shown that vitamin D is necessary for lipid synthesis and adipose tissue formation during development. In VDR-knockout mice, the absence

of vitamin D action showed substantial protective effects against high-fat diet-induced adipose tissue accumulation [78, 79]. On the other hand, in another knock-in model, mice with overexpressed human VDR in adipose tissue developed obesity even if fed with a standard diet [80]. Further investigations into this mechanism revealed that vitamin D potentiates the differentiation of subcutaneous preadipocytes via the VDR pathway, and at the same time, the expression level of VDR dropped significantly when adipocytes reached their full maturity [81]. Nevertheless, vitamin D was also reported to alleviate atherosclerosis progressing in patients by preventing foam cell formation [82] and suppressing the oxidative stress in blood vessel endothelial cells [83]. However, the roles of vitamin D in lipid metabolism described by above-mentioned studies s are not necessarily associated with stabilizing the blood lipid profiles. So far, although clinical studies have identified the protective role of vitamin D in patients with various backgrounds and differential causes of dyslipidemia [52, 53], the primary medical function of vitamin D, which improves calcium absorption, is not likely to relate to its positive role in treating dyslipidemia, as a study showed that sole calcium supplements did not promise a better blood lipid profile [84]. To date, studies revealing the mechanism of these clinical results are scarce. Nevertheless, a recent study established a connection by proving that 25(OH)D_3 inhibits cholesterol and fatty acids biosynthesis by preventing SREBPs from maturing in a VDR-independent pattern [70], but this result is not reproducible in primary hepatocytes and adipocytes of the present study. This may be due to the vast metabolic variance between highly differentiated primary cells and immortalized cells (CHO cell line) in the mentioned report. Another study This understanding is consistent with the lack of VDR expression in primary cultured hepatocytes in the western blot results of the present study. Nevertheless, the VDR activation-induced *Insig2* expression increase is reproduced in this study. Insig2

is an essential regulator of cholesterol biosynthesis that not only prevents SREBPs from entering the cell nucleus to suppress their transcription function [67] but also facilitates HMGCR degradation [85]. A functional vitamin D response element has been localized in the promoter of the mouse *Insig2* [86], and further comparisons between rodent and human *Insig2* promoters have confirmed that this vitamin D response element is also included in the human genome [87]. Although, in contrast to the liver, the role of the muscle tissues is minor in the essentiality of cholesterol production and balance, cholesterol production in peripheral tissues should not be overlooked. A report has shown myopathy development in skeletal muscle-specific *Hmgcr* knockout mice [88]. Additionally, humans with skeletal muscle *Hmgcr* deficiency easily develop myopathy [89]. Furthermore, as VDR is widely expressed in tissues throughout the body [47], vitamin D may improve the blood cholesterol condition by limiting tissue self-biosynthesis of cholesterol and simultaneously inducing cholesterol absorption in peripheral tissues.

Conclusion

In this research, using drug repurposing based on clinical big data as an updated approach, vitamin D is identified as promising to prevent olanzapine-induced dyslipidemia.

In the first chapter, analysis of the FAERS database, olanzapine treatment was found to be highly related to the occurrence of dyslipidemia, while vitamin D was negatively related to the occurrence of olanzapine-induced dyslipidemia. This finding has been further confirmed in the JMDC database, as blood lipid profiles have been significantly improved in patients who were supplemented with vitamin D while under an olanzapine treatment, and a clear causal relationship has been established between vitamin D supplementations and lipid profile improvements. Furthermore, the analysis of the NUSW database provided continuous tracing on the lipid profile deteriorations by olanzapine and the preventing effects of vitamin D in units of days, which improved the precision of the analysis.

In the second chapter, olanzapine-induced dyslipidemia was confirmed by broken blood cholesterol homeostasis in mouse models. Further investigations using cultured hepatocyte, adipocyte, and C2C12 cells found that the vitamin D metabolites suppressed the expression of cholesterol-biosynthesis-related genes in C2C12 cells. The bioactive form of vitamin D ($1,25(OH)_2D_3$) displayed a high suppressive capability than the inactive form of vitamin D ($25(OH)D_3$). Also, VDR protein is highly enriched in C2C12 cells but not in hepatocytes and adipocytes. Aside from vitamin D natural metabolites, another artificial VDR partial agonist, ZK159222, displayed similar capability in suppressive effects.

This study is ready to be a demonstration of the potential of the clinical-big-data-based drug repurposing approach in discovering the new treating effects of existing drugs. Nevertheless, there are still unclarified details in the mechanisms of olanzapine-induced dyslipidemia, and future work will focus on deciphering the enigmas of this aspect.

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Reference

1. Saha, S., et al., *A systematic review of the prevalence of schizophrenia.* PLoS Med, 2005. **2**(5): p. e141.
2. Divac, N., et al., *Second-generation antipsychotics and extrapyramidal adverse effects.* Biomed Res Int, 2014. **2014**: p. 656370.
3. Kochi, K., et al., *Trends in antipsychotic prescriptions for japanese outpatients during 2006-2012: A descriptive epidemiological study.* Pharmacoepidemiol Drug Saf, 2017. **26**(6): p. 642–656.
4. Lieberman, J.A., et al., *Effectiveness of antipsychotic drugs in patients with chronic schizophrenia.* N Engl J Med, 2005. **353**(12): p. 1209–23.
5. Li, R., et al., *Effects of olanzapine treatment on lipid profiles in patients with schizophrenia: A systematic review and meta-analysis.* Sci Rep, 2020. **10**(1): p. 17028.
6. Wang, J., et al., *Olanzapine-induced weight gain plays a key role in the potential cardiovascular risk: Evidence from heart rate variability analysis.* Sci Rep, 2014. **4**: p. 7394.
7. Czekalla, J., S. Kollack-Walker, and C.M. Beasley, Jr., *Cardiac safety parameters of olanzapine: Comparison with other atypical and typical antipsychotics.* J Clin Psychiatry, 2001. **62 Suppl 2**: p. 35-40.
8. Correll, C.U., et al., *Cardiovascular and cerebrovascular risk factors and events associated with second-generation antipsychotic compared to antidepressant use in a non-elderly adult sample: Results from a claims-based inception cohort study.* World Psychiatry, 2015. **14**(1): p. 56–63.
9. Brown, S., H. Inskip, and B. Barraclough, *Causes of the excess mortality of schizophrenia.* Br J Psychiatry, 2000. **177**: p. 212–7.
10. Yan, H., J.D. Chen, and X.Y. Zheng, *Potential mechanisms of atypical antipsychotic-induced hypertriglyceridemia.* Psychopharmacology (Berl), 2013. **229**(1): p. 1–7.
11. Farha, M.A. and E.D. Brown, *Drug repurposing for antimicrobial discovery.* Nat Microbiol, 2019. **4**(4): p. 565-577.
12. Pushpakom, S., et al., *Drug repurposing: Progress, challenges and recommendations.* Nat Rev Drug Discov, 2019. **18**(1): p. 41-58.
13. Parvathaneni, V., et al., *Drug repurposing: A promising tool to accelerate the drug discovery process.* Drug Discov Today, 2019. **24**(10): p. 2076-2085.
14. Narula, P.K., et al., *Topiramate for prevention of olanzapine associated weight gain and metabolic dysfunction in schizophrenia: A double-blind, placebo-controlled trial.* Schizophr Res, 2010. **118**(1-3): p. 218–23.
15. Larsen, J.R., et al., *Effect of liraglutide treatment on prediabetes and overweight or obesity in clozapine- or olanzapine-treated patients with schizophrenia spectrum disorder: A randomized clinical trial.* JAMA Psychiatry, 2017. **74**(7): p. 719–728.
16. Chen, C.H., et al., *Metformin for metabolic dysregulation in schizophrenic patients treated with olanzapine.* Prog Neuropsychopharmacol Biol Psychiatry, 2008. **32**(4): p. 925–31.
17. Kanagasundaram, P., et al., *Pharmacological interventions to treat antipsychotic-induced dyslipidemia in schizophrenia patients: A systematic review and meta analysis.* Front Psychiatry, 2021. **12**: p. 642403.
18. Kaneko, S. and T. Nagashima, *Drug repositioning and target finding based on clinical evidence.* Biol Pharm Bull, 2020. **43**(3): p. 362–365.
19. *"Fda adverse event reporting system (faers).".* Available from: http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/.

20. Nagashima, T., et al., *Prevention of antipsychotic-induced hyperglycaemia by vitamin d: A data mining prediction followed by experimental exploration of the molecular mechanism.* Sci Rep, 2016. **6**: p. 26375.
21. Siswanto, S., et al., *Drug repurposing prediction and validation from clinical big data for the effective treatment of interstitial lung disease.* Front Pharmacol, 2021. **12**: p. 635293.
22. Nagaoka, K., et al., *Striatal trpv1 activation by acetaminophen ameliorates dopamine d2 receptor antagonist-induced orofacial dyskinesia.* JCI Insight, 2021. **6**(10).
23. Nagashima, T., et al., *Identifying antidepressants less likely to cause hyponatremia: Triangulation of retrospective cohort, disproportionality, and pharmacodynamic studies.* Clin Pharmacol Ther, 2022. **111**(6): p. 1258–1267.
24. Banda, J.M., et al., *A curated and standardized adverse drug event resource to accelerate drug safety research.* Sci Data, 2016. **3**: p. 160026.
25. Alatawi, Y.M. and R.A. Hansen, *Empirical estimation of under-reporting in the u.S. Food and drug administration adverse event reporting system (faers).* Expert Opin Drug Saf, 2017. **16**(7): p. 761–767.
26. Jann, M.W., et al., *Pharmacokinetics and pharmacodynamics of clozapine.* Clin Pharmacokinet, 1993. **24**(2): p. 161–76.
27. DeVane, C.L. and C.B. Nemeroff, *Clinical pharmacokinetics of quetiapine: An atypical antipsychotic.* Clin Pharmacokinet, 2001. **40**(7): p. 509–22.
28. Callaghan, J.T., et al., *Olanzapine. Pharmacokinetic and pharmacodynamic profile.* Clin Pharmacokinet, 1999. **37**(3): p. 177–93.
29. Bohon, T.M. and M.A. Goolsby, *The role of vitamin d supplements in women's health.* Clin Med Insights Womens Health, 2013. **6**: p. 67–70.
30. Kryzhanovskaya, L., et al., *Olanzapine versus placebo in adolescents with schizophrenia: A 6-week, randomized, double-blind, placebo-controlled trial.* J Am Acad Child Adolesc Psychiatry, 2009. **48**(1): p. 60–70.
31. Kaviyani, S., et al., *Therapeutic impact of cholecalciferol in patients with psychiatric disorders receiving olanzapine.* Journal of Parathyroid Disease, 2017. **6**(1): p. 19–22.
32. Carr, M.C., *The emergence of the metabolic syndrome with menopause.* J Clin Endocrinol Metab, 2003. **88**(6): p. 2404–11.
33. Citrome, L., et al., *A commentary on the efficacy of olanzapine for the treatment of schizophrenia: The past, present, and future.* Neuropsychiatr Dis Treat, 2019. **15**: p. 2559-2569.
34. Bymaster, F.P., et al., *Radioreceptor binding profile of the atypical antipsychotic olanzapine.* Neuropsychopharmacology, 1996. **14**(2): p. 87-96.
35. Moore, N.A., et al., *The behavioral pharmacology of olanzapine, a novel "atypical" antipsychotic agent.* J Pharmacol Exp Ther, 1992. **262**(2): p. 545-51.
36. Fuller, R.W. and H.D. Snoddy, *Neuroendocrine evidence for antagonism of serotonin and dopamine receptors by olanzapine (ly170053), an antipsychotic drug candidate.* Res Commun Chem Pathol Pharmacol, 1992. **77**(1): p. 87-93.
37. Kaar, S.J., et al., *Antipsychotics: Mechanisms underlying clinical response and side-effects and novel treatment approaches based on pathophysiology.* Neuropharmacology, 2020. **172**: p. 107704.
38. Uhlen, M., et al., *Proteomics. Tissue-based map of the human proteome.* Science, 2015. **347**(6220): p. 1260419.
39. Zhu, W., et al., *Metformin ameliorates hepatic steatosis induced by olanzapine through inhibiting lxralpha/pcsk9 pathway.* Sci Rep, 2022. **12**(1): p. 5639.

40. Liu, X.M., et al., *Simvastatin improves olanzapine-induced dyslipidemia in rats through inhibiting hepatic mtor signaling pathway.* Acta Pharmacol Sin, 2019. **40**(8): p. 1049–1057.
41. Jiang, T., et al., *Up-regulation of hepatic fatty acid transporters and inhibition/down-regulation of hepatic octn2 contribute to olanzapine-induced liver steatosis.* Toxicol Lett, 2019. **316**: p. 183–193.
42. Chen, C.H., et al., *Atypical antipsychotic drug olanzapine deregulates hepatic lipid metabolism and aortic inflammation and aggravates atherosclerosis.* Cell Physiol Biochem, 2018. **50**(4): p. 1216–1229.
43. Yang, L.H., et al., *Olanzapine induces srebp-1-related adipogenesis in 3t3-l1 cells.* Pharmacol Res, 2007. **56**(3): p. 202-8.
44. Lauressergues, E., et al., *Antipsychotic drug action on srebps-related lipogenesis and cholesterogenesis in primary rat hepatocytes.* Naunyn Schmiedebergs Arch Pharmacol, 2010. **381**(5): p. 427-39.
45. Kristiana, I., et al., *Antipsychotic drugs upregulate lipogenic gene expression by disrupting intracellular trafficking of lipoprotein-derived cholesterol.* Pharmacogenomics J, 2010. **10**(5): p. 396-407.
46. Bikle, D. and S. Christakos, *New aspects of vitamin d metabolism and action - addressing the skin as source and target.* Nat Rev Endocrinol, 2020. **16**(4): p. 234-252.
47. Remelli, F., et al., *Vitamin d deficiency and sarcopenia in older persons.* Nutrients, 2019. **11**(12).
48. Wan, Z., et al., *Vitamin d status, genetic factors, and risk of cardiovascular disease among individuals with type 2 diabetes: A prospective study.* Am J Clin Nutr, 2022.
49. Pereira-Santos, M., et al., *Obesity and vitamin d deficiency: A systematic review and meta-analysis.* Obes Rev, 2015. **16**(4): p. 341-9.
50. Jiang, X., et al., *Vitamin d deficiency is associated with dyslipidemia: A cross-sectional study in 3788 subjects.* Curr Med Res Opin, 2019. **35**(6): p. 1059-1063.
51. Drincic, A.T., et al., *Volumetric dilution, rather than sequestration best explains the low vitamin d status of obesity.* Obesity (Silver Spring), 2012. **20**(7): p. 1444-8.
52. Mirhosseini, N., J. Rainsbury, and S.M. Kimball, *Vitamin d supplementation, serum 25(oh)d concentrations and cardiovascular disease risk factors: A systematic review and meta-analysis.* Front Cardiovasc Med, 2018. **5**: p. 87.
53. Dibaba, D.T., *Effect of vitamin d supplementation on serum lipid profiles: A systematic review and meta-analysis.* Nutr Rev, 2019. **77**(12): p. 890–902.
54. Gascon-Barre, M., et al., *The normal liver harbors the vitamin d nuclear receptor in nonparenchymal and biliary epithelial cells.* Hepatology, 2003. **37**(5): p. 1034–42.
55. Charni-Natan, M. and I. Goldstein, *Protocol for primary mouse hepatocyte isolation.* STAR Protoc, 2020. **1**(2): p. 100086.
56. Jung, Y., M. Zhao, and K.J. Svensson, *Isolation, culture, and functional analysis of hepatocytes from mice with fatty liver disease.* STAR Protoc, 2020. **1**(3): p. 100222.
57. Galmozzi, A., B.P. Kok, and E. Saez, *Isolation and differentiation of primary white and brown preadipocytes from newborn mice.* J Vis Exp, 2021(167).
58. Lequeux, C., et al., *Optimization of a culture medium for the differentiation of preadipocytes into adipocytes in a monolayer.* Biomed Mater Eng, 2009. **19**(4-5): p. 283–91.
59. Cock, P.J., et al., *The sanger fastq file format for sequences with quality scores, and the solexa/illumina fastq variants.* Nucleic Acids Res, 2010. **38**(6): p. 1767–71.
60. Langmead, B. and S.L. Salzberg, *Fast gapped-read alignment with bowtie 2.* Nat Methods, 2012. **9**(4): p. 357–9.

61. Kim, D., B. Langmead, and S.L. Salzberg, *Hisat: A fast spliced aligner with low memory requirements.* Nat Methods, 2015. **12**(4): p. 357–60.
62. Li, B. and C.N. Dewey, *Rsem: Accurate transcript quantification from rna-seq data with or without a reference genome.* BMC Bioinformatics, 2011. **12**: p. 323.
63. Hayamizu, T.F., et al., *The adult mouse anatomical dictionary: A tool for annotating and integrating data.* Genome Biol, 2005. **6**(3): p. R29.
64. Audic, S. and J.M. Claverie, *The significance of digital gene expression profiles.* Genome Res, 1997. **7**(10): p. 986–95.
65. Ohashi, K., et al., *Trpv4 is functionally expressed in oligodendrocyte precursor cells and increases their proliferation.* Pflugers Arch, 2018. **470**(5): p. 705–716.
66. Faeh, D., et al., *Effect of fructose overfeeding and fish oil administration on hepatic de novo lipogenesis and insulin sensitivity in healthy men.* Diabetes, 2005. **54**(7): p. 1907–13.
67. Yabe, D., M.S. Brown, and J.L. Goldstein, *Insig-2, a second endoplasmic reticulum protein that binds scap and blocks export of sterol regulatory element-binding proteins.* Proc Natl Acad Sci U S A, 2002. **99**(20): p. 12753–8.
68. DeBose-Boyd, R.A., *Feedback regulation of cholesterol synthesis: Sterol-accelerated ubiquitination and degradation of hmg coa reductase.* Cell Res, 2008. **18**(6): p. 609–21.
69. Horton, J.D., et al., *Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct srebp target genes.* Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12027–32.
70. Asano, L., et al., *Vitamin d metabolite, 25-hydroxyvitamin d, regulates lipid metabolism by inducing degradation of srebp/scap.* Cell Chem Biol, 2017. **24**(2): p. 207–217.
71. Teske, K.A., O. Yu, and L.A. Arnold, *Inhibitors for the vitamin d receptor-coregulator interaction.* Vitam Horm, 2016. **100**: p. 45–82.
72. Larrey, D. and M.-P. Ripault, *Hepatotoxicity of psychotropic drugs and drugs of abuse*, in *Drug-induced liver disease*. 2013. p. 443–462.
73. Morlan-Coarasa, M.J., et al., *Incidence of non-alcoholic fatty liver disease and metabolic dysfunction in first episode schizophrenia and related psychotic disorders: A 3-year prospective randomized interventional study.* Psychopharmacology (Berl), 2016. **233**(23-24): p. 3947-3952.
74. Huang, J., et al., *Increased appetite plays a key role in olanzapine-induced weight gain in first-episode schizophrenia patients.* Frontiers in Pharmacology, 2020. **11**.
75. Mathews, J., et al., *Neural correlates of weight gain with olanzapine.* Arch Gen Psychiatry, 2012. **69**(12): p. 1226–37.
76. Kim, S.F., et al., *From the cover: Antipsychotic drug-induced weight gain mediated by histamine h1 receptor-linked activation of hypothalamic amp-kinase.* Proc Natl Acad Sci U S A, 2007. **104**(9): p. 3456–9.
77. He, M., et al., *Hypothalamic histamine h1 receptor-ampk signaling time-dependently mediates olanzapine-induced hyperphagia and weight gain in female rats.* Psychoneuroendocrinology, 2014. **42**: p. 153–64.
78. Wong, K.E., et al., *Involvement of the vitamin d receptor in energy metabolism: Regulation of uncoupling proteins.* Am J Physiol Endocrinol Metab, 2009. **296**(4): p. E820–8.
79. Narvaez, C.J., et al., *Lean phenotype and resistance to diet-induced obesity in vitamin d receptor knockout mice correlates with induction of uncoupling protein-1 in white adipose tissue.* Endocrinology, 2009. **150**(2): p. 651–61.

80. Wong, K.E., et al., *Targeted expression of human vitamin d receptor in adipocytes decreases energy expenditure and induces obesity in mice.* J Biol Chem, 2011. **286**(39): p. 33804–10.
81. Nimitphong, H., et al., *25-hydroxyvitamin d(3) and 1,25-dihydroxyvitamin d(3) promote the differentiation of human subcutaneous preadipocytes.* PLoS One, 2012. **7**(12): p. e52171.
82. Oh, J., et al., *1,25(oh)2 vitamin d inhibits foam cell formation and suppresses macrophage cholesterol uptake in patients with type 2 diabetes mellitus.* Circulation, 2009. **120**(8): p. 687–98.
83. Kassi, E., et al., *Role of vitamin d in atherosclerosis.* Circulation, 2013. **128**(23): p. 2517–31.
84. Reid, I.R., et al., *Effects of calcium supplementation on lipids, blood pressure, and body composition in healthy older men: A randomized controlled trial.* Am J Clin Nutr, 2010. **91**(1): p. 131–9.
85. Jo, Y., et al., *Sterol-induced degradation of hmg coa reductase depends on interplay of two insigs and two ubiquitin ligases, gp78 and trc8.* Proc Natl Acad Sci U S A, 2011. **108**(51): p. 20503–8.
86. Lee, S., et al., *Identification of a functional vitamin d response element in the murine insig-2 promoter and its potential role in the differentiation of 3t3-l1 preadipocytes.* Mol Endocrinol, 2005. **19**(2): p. 399–408.
87. Fernandez-Alvarez, A., et al., *Characterization of the human insulin-induced gene 2 (insig2) promoter: The role of ets-binding motifs.* J Biol Chem, 2010. **285**(16): p. 11765–74.
88. Osaki, Y., et al., *Skeletal muscle-specific hmg-coa reductase knockout mice exhibit rhabdomyolysis: A model for statin-induced myopathy.* Biochem Biophys Res Commun, 2015. **466**(3): p. 536–40.
89. Lorenzo-Villalba, N., E. Andres, and A. Meyer, *Chronic onset form of anti-hmg-coa reductase myopathy.* Eur J Case Rep Intern Med, 2021. **8**(7): p. 002672.