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Original Research

Serum 25-hydroxycholesterol levels are increased in patients with coronavirus disease 2019†

Takumi Asano, BA, Tetsuji Wakabayashi, MD, PhD, Yasuyuki Kondo, MD, Kenta Okada, MD, PhD, Daisuke Yamamuro, PhD, Yukiko Koga, MD, Kiyonori Oka, MD, Momoe Sakurai, MD, Nagisa Sawayama, MD, Manabu Takahashi, MD, PhD, Hiroaki Okazaki, MD, PhD, Ken Ebihara, MD, PhD, Kensuke Minami, MD, Yuji Morisawa, MD, Shuji Hatakeyama, MD, PhD, Masami Matsumura, MD, PhD, Shun Ishibashi, MD, PhD*

Division of Endocrinology and Metabolism, Department of Internal Medicine, Jichi Medical University, Shimotsuke, Tochigi, Japan (Drs Asano, Wakabayashi, Kondo, Okada, Yamamuro, Koga, Oka, Sakurai, Sawayama, Takahashi, Okazaki, Ebihara and Ishibashi); Division of Infectious Diseases, Jichi Medical University Hospital, Shimotsuke, Tochigi, Japan (Drs Minami and Morisawa); Division of General Medicine, Center for Community Medicine, Jichi Medical University, Shimotsuke, Tochigi, Japan (Drs Hatakeyama and Matsumura)

KEYWORDS
COVID-19; Oxysterol; 25-Hydroxycholesterol; Cholesterol 25-hydroxylase; Serum biomarker

Background: 25-hydroxycholesterol (25HC), produced by cholesterol 25-hydroxylase (CH25H) in macrophages, has been reported to inhibit the replication of viral pathogens such as severe acute respiratory syndrome coronavirus-2. Also, CH25H expression in macrophages is robustly induced by interferons (IFNs).

Objective: To better understand the serum level increase of 25HC in coronavirus disease 2019 (COVID-19) and how it relates to the clinical picture.

Methods: We measured the serum levels of 25HC and five other oxysterols in 17 hospitalized COVID-19 patients.

Results: On admission, 25HC and 27-hydroxycholesterol (27HC) serum levels were elevated; however, 7-ketocholesterol (7KC) levels were lower in patients with COVID-19 than in the healthy controls. There was no significant correlation between 25HC serum levels and disease severity markers, such as interferon-gamma (IFN-γ) and interleukin 6. Dexamethasone effectively suppressed cholesterol 25-hydroxylase (CH25H) mRNA expression in RAW 264.7 cells, a murine leukemia macrophage cell line, with or without lipopolysaccharide or IFNs; therefore, it might mitigate the increasing effects of COVID-19 on the serum levels of 25HC.

† TA and TW contributed equally to this work.
* Corresponding author. Shun Ishibashi at Division of Endocrinology and Metabolism, Department of Internal Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi, 329-0031, Japan
E-mail address: ishibashi@jichi.ac.jp (S. Ishibashi).
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Introduction

Coronavirus disease 2019 (COVID-19) is a rapidly spreading transmissible disease caused by the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). COVID-19 has become a major pandemic since its discovery in December 2019. As of April 2022, there were approximately 500 million confirmed cases, including over 6 million deaths, and the incidence continues to rise rapidly, although vaccination coverage has increased. There are few specific effective treatments in most cases, hence the need to identify a new therapeutic strategy.

Oxysterols, which are selective cholesterol oxidation products, are involved in various pathological processes, including infection and inflammation. Particularly, 25-hydroxycholesterol (25HC) inhibits the replication of various viral pathogens. Recently, SARS-CoV-2 infection has been observed to increase 25HC concentrations by inducing the expression of cholesterol 25-hydroxylase (CH25H) in macrophages, thereby restricting SARS-CoV-2 replication in vitro.

CH25H is an interferon (IFN)-stimulated gene (ISG) that is transcriptionally regulated via the signal transducer and activator of the transcription 1 (STAT1) pathway. Reportedly, CH25H gene expression in murine macrophages is induced by lipopolysaccharides (LPS) and this LPS-mediated expression of CH25H is dependent on TIR-domain-containing adapter-inducing interferon-β (TRIF), production of type I IFNs, and signaling through the interferon production regulator (IFNR)/Janus kinase (JAK)/STAT1 pathway. In addition to CH25H in macrophages, 25HC is produced by cytochrome P450 3A4 (CYP3A4) and CYP27B1 in the liver, CYP46A1 in the brain and autoxidation. However, it is mostly unknown whether infectious diseases with an elevation of LPS or IFNs are associated with elevated 25HC serum levels, thereby influencing the clinical courses of infectious diseases in humans.

Zu et al. reported elevation of serum 25HC levels in a 73-year-old female COVID-19 patient. Marcello et al. reported that serum 25HC levels were increased in pausi-asymptomatic, but not in moderate or severe patients with COVID-19. In addition, they reported that serum levels of 27-hydroxycholesterol (27HC), which has also in vitro inhibitory activity against SARS-CoV-2, were significantly decreased in COVID-19 patients.

We measured the serum concentrations of the following oxysterols in patients with severe COVID-19 who were admitted to our hospital to determine the relationship between the serum levels of oxysterols and the clinical course of COVID-19: 4β-hydroxycholesterol (4βHC), 7α-hydroxycholesterol (7αHC), 7-ketocholesterol (7KC), 24s-hydroxycholesterol (24SHC), 25HC, and 27HC. The results revealed that the serum concentrations of 25HC and 27HC were significantly increased, whereas that of 7KC was significantly decreased in COVID-19 patients.

Materials and methods

Participants

The study participants included 17 COVID-19 patients hospitalized at Jichi Medical University Hospital between December 2020 and September 2021. Disease status was classified into four stages following the guidelines issued by the Ministry of Health, Labour, and Welfare, Japan: mild, asymptomatic carrier or coughing only; moderate I, pneumonia and/or breathlessness but not respiratory insufficiency; moderate II, respiratory insufficiency requiring supplemental oxygen; and severe, respiratory failure requiring ventilation or intensive care unit admission. Sixteen patients were diagnosed with severe COVID-19 at other hospitals and transferred to our hospital for more intensive treatment. In analyzing serum oxysterols, venous blood samples were collected in the morning after fasting for over 10 h within 2 days after admission. Serum was separated by centrifugation and stored at -80°C until further analyses. The following laboratory tests were performed upon hospital admission: complete blood count, D-dimer, C-reactive protein (CRP), serum albumin, serum creatinine, blood urea nitrogen, total bilirubin, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, and serum uric acid concentration. Also, total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were estimated using the Friedewald equation.

Control data were collected from 206 healthy volunteers who visited Utsunomiya Higashi Hospital for health check-ups as previously described.
The ethics committees approved the experimental protocol of the Jichi Medical University and Utsunomiya Higashi Hospital. Written informed consent was obtained from all the participants to use the serum samples in the study, and the experimental procedures were conducted in accordance with the ethical standards of the Declaration of Helsinki.

Quantification of serum oxysterols

Serum oxysterols, including 25HC, 4βHC, 7αHC, 7KC, 24sHC, and 27HC, were quantified using liquid chromatograph-mass spectrometry (LC-MS/MS) as described previously.13,24

Enzyme-linked immune-sorbent assay (ELISA)

The serum samples were analyzed for cytokine concentrations using ELISA MAX Standard Sets (BioLegend; human IL-6, #430501; human IFN-γ, #430101), according to the manufacturer’s instructions.

Cell Culture Experiments

Blood was collected from healthy volunteers to isolate peripheral blood mononuclear cells (PBMCs) using the EasySep Direct Human Monocyte Isolation Kit (VERITAS, #ST-19669) according to the manufacturer’s instructions. PBMCs were cultured in RPMI1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics with 50 ng/ml human macrophage colony-stimulating factor (M-CSF) (FUJIFILM Wako, #133-13611) for 6 days to induce differentiation into macrophages (human monocyte-derived macrophages (HMDMs)). To measure CH25H expression, HMDMs were treated with 100 ng/mL LPS (LPS; Sigma, #L-2630), 100 ng/mL IFN-α (Sigma, #SRP4596), and 100 ng/mL IFN-γ (Sigma, #SRP3058) for 6 h.

The murine leukemia macrophage cell line RAW 264.7, and the human hepatoma cell line HepG2, originally obtained from the American Type Culture Collection were cultured in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% (v/v) FBS and antibiotics. In measuring gene expression, RAW cells and HepG2 cells were treated with 100 ng/mL LPS, 100 ng/mL IFN-α (mouse IFN-α; BioLegend, #752804), and 100 ng/mL IFN-γ (mouse IFN-γ; FUJIFILM Wako, #094-04701) for 6 h with or without 1 μM DEX (Sigma, #D1756).

Quantitative real-time polymerase chain reaction (PCR)

Quantitative real-time PCR was performed as described previously.25 The primer sets used for real-time PCR are listed in Supplementary Table 1.

Statistics

All the results are presented as mean ± SD. The Student’s t-test was used to compare two unpaired groups, and the one-way ANOVA was used for multiple comparison. When ANOVA results were statistically significant (i.e., p<0.05), individual comparisons were made with the Tukey posthoc test. The Wilcoxon signed-rank test was used to compare the two paired parameters, and the Spearman’s rank correlation test evaluated the degree of association between two paired variables. The cut-off values and the area under the curve (AUC) were determined by receiver operating characteristic (ROC) analysis. All analyses were performed using GraphPad Prism version 9.0 (GraphPad Prism software). Statistical significance was set at a p-value of <0.05.

Results

Clinical Features of COVID-19 patients

The clinical characteristics of the patients are shown in Tables 1 and 2. All the COVID-19 patients recovered and were discharged. Fifteen of the 17 patients had severe symptoms, 1 had moderate, and the last had mild symptoms. Twelve patients were obese (body mass index (BMI)>25), 6 were hypertensive, 3 were diabetic, and 1 had dyslipidemia. Sixteen patients with moderate-to-severe symptoms were treated with DEX. In 13 patients, blood samples were collected after the DEX therapy was initiated.

As shown in Table 2, BMI, white blood cell (WBC), urea nitrogen (UN), and TG were significantly higher. However, UA, TC, HDL-C, and LDL-C levels were significantly lower in COVID-19 patients on admission than in the healthy controls. Furthermore, neutrophil counts, fibrin/fibrinogen degradation products (FDP), D-dimer, CRP, procalcitonin, ferritin, lactate dehydrogenase (LDH), and creatine phosphokinase (CPK) were high. In contrast, lymphocyte counts and albumin levels were lower in COVID-19 patients on admission than the reference values.

Serum oxysterol concentrations in COVID-19 patients

The serum concentrations of oxysterols in the COVID-19 patients were compared with those of the controls (Fig. 1). There were positive correlations between serum TC and all the oxysterols in the healthy controls (Supplementary Fig. 1); therefore, oxysterol concentrations were corrected for TC concentrations to reduce the bias of hyperlipidemia.

The concentrations of 25HC, 24SHC, and 27HC were significantly elevated by +63%, +18%, and +61%, respectively, in COVID-19 patients compared to the controls. In contrast, 7KC concentrations in COVID-19 patients were significantly decreased by -28% compared to the controls. However, 4βHC and 7αHC concentrations were not significantly different between COVID-19 patients and the controls. There was no significant difference in 24SHC concentrations without TC correction between the COVID-19 patients and controls (Supplementary Fig. 2).
Table 1  Summary of clinical courses of the patients with COVID-19.

| #  | Age | Sex | BMI                  | Comorbidities                      | Severity | B | D | P | Agents               | HP |
|----|-----|-----|----------------------|------------------------------------|----------|----|---|---|----------------------|----|
| 1  | 72  | M   | 25.7                 | Hypertension                       | Severe   | 13 | 10| 5 | Remdesivir           | 22 |
| 2  | 67  | M   | 22.0                 | Myelodysplastic syndromes          | Mild     | 8  |    |   |                      | 24 |
| 3  | 63  | M   | 27.0                 | Hypertension, Smoking              | Severe   | 9  | 5 | 6 | Remdesivir, Tocilizumab | 22 |
| 4  | 59  | M   | 38.8                 | Chronic liver disease, Sleep apnea syndrome | Severe   | 3  | 2 | 19 | Remdesivir           | 20 |
| 5  | 73  | F   | 17.1                 |                                    | Severe   | 11 | 8 | 10|                      | 23 |
| 6  | 46  | M   | 24.4                 | Dyslipidemia                       | Severe   | 10 | 7 | 7 | Tocilizumab          | 21 |
| 7  | 60  | M   | 20.6                 | Chronic heart disease, Chronic kidney disease | Severe   | 7  | 7 | 10|                      | 32 |
| 8  | 35  | M   | 31.0                 |                                    | Severe   | 7  | 5 | 10|                      | 19 |
| 9  | 50  | M   | 26.0                 | Hypertension, Diabetes Mellitus, Smoking | Severe  | 14 | 7 | 7 | Remdesivir, Tocilizumab, Baricitinib | 28 |
| 10 | 54  | M   | 36.4                 | Hypertension, Sleep apnea syndrome | Severe   | 12 | 9 | 10| Remdesivir, Baricitinib | 23 |
| 11 | 56  | M   | 36.0                 | Hypertension                       | Severe   | 15 | 8 | 10| Remdesivir, Tocilizumab | 29 |
| 12 | 48  | M   | 32.9                 | Diabetes Mellitus                  | Severe   | 14 | 11 | 7 | Tocilizumab          | 20 |
| 13 | 35  | M   | 29.4                 |                                    | Severe   | 11 | 9 | 10| Remdesivir, Baricitinib | 22 |
| 14 | 43  | M   | 29.3                 | Smoking                            | Severe   | 13 | 13| 10|                      | 28 |
| 15 | 61  | M   | 22.2                 |                                    | Moderate II | 4 | 2 | 11| Remdesivir          | 12 |
| 16 | 61  | M   | 25.6                 | Hypertension, Diabetes Mellitus    | Severe   | 6  | 6 | 9 | Remdesivir, Tocilizumab, Baricitinib | 14 |
| 17 | 50  | M   | 25.7                 |                                    | Severe   | 11 | 8 | 9 | Tocilizumab          | 22 |

Abbreviations: B (blood collection), the period from onset to the day when blood was collected (days); D, the period from onset to the day when DEX was initiated (days); P, the duration of treatment with DEX (days); HP (hospitalization), duration of hospitalization (days). M, male; F, female.

Table 2  Clinical Features of the patients with COVID-19.

| Background and Clinical Data | Healthy volunteers (n=206) | COVID-19 (n=17) | Reference values* | p   |
|-----------------------------|---------------------------|----------------|-------------------|-----|
| Gender (male/female)        | 132/74                    | 16/1           | -                 | -   |
| Age (years)                 | 47.0±9.3                  | 54.9±11.4      | -                 | -   |
| BMI (Kg/m²)                 | 22.7±3.1                  | 27.7±5.9       | -                 | <0.0001 |
| WBC (×10⁹/μL)               | 5.4±1.4                   | 7.7±3.6        | 5.9±2.6           | <0.0005 |
| Neutrophil (×10⁹/μL)        | NA                        | 6.4±3.2        | 3.7±2.4           | -   |
| Lymphocyte (×10⁹/μL)        | NA                        | 0.7±0.3        | 2.5±1.6           | -   |
| Hb (g/dL)                   | 14.3±1.5                  | 13.2±2.5       | male; 15.2±1.6; female; 13.2±1.6 | NS |
| Plt (×10⁹/μL)               | 24.3±4.9                  | 22.4±10.5      | 25.3±9.5          | NS |
| FDP                         | NA                        | 15.4±18.8      | < 5.0             | -   |
| D-dimer (μg/mL)             | NA                        | 8.6±19.2       | < 1.0             | -   |
| CRP (mg/dL)                 | NA                        | 5.8±4.6        | 0.07±0.07         | -   |
| Procalcitonin (ng/mL)       | NA                        | 1.3±4.2        | < 0.046           | -   |
| Ferritin (ng/mL)            | NA                        | 890.8±330.9    | male; 145.0±132.0; female; 78.5±73.5 | NS |
| Albumin (g/dL)              | NA                        | 3.0±0.5        | 4.6±0.5           | -   |
| Total bilirubin (mg/dL)     | NA                        | 0.6±0.3        | 1.0±0.6           | -   |
| AST (IU/L)                  | 21.1±7.7                  | 46.8±36.3      | 22.0±9.0          | <0.0001 |
| ALT (IU/L)                  | 22.1±17.1                 | 57.2±54.0      | male; 26.0±16.0; female; 15.0±8.0 | <0.0001 |
| γ-GTP (IU/L)                | 28.6±24.7                 | 94.2±99.8      | male; 38.5±25.5; female; 20.5±11.5 | <0.0001 |
| LDH (U/L)                   | NA                        | 385.9±126.4    | 173.0±49.0        | -   |
| CPK (U/L)                   | NA                        | 567.9±142.4    | male; 153.5±94.5; female; 97.0±56.0 | -   |
| UN (mg/dL)                  | 12.8±2.9                  | 26.7±14.0      | 14.0±6.0          | <0.0001 |
| Creatinine (mg/dL)          | 0.8±0.2                   | 1.6±2.8        | male; 0.9±0.2; female; 0.6±0.2 | NS |
| UA (mg/dL)                  | 5.6±1.2                   | 3.9±2.0        | male; 5.8±2.1; female; 4.1±1.5 | <0.0005 |
| K-L6 (μL)                   | NA                        | 438.1±227.1    | < 500.0           | -   |
| Total Cholesterol (mg/dL)   | 202.4±32.9                | 165.8±39.6     | 195.0±53.0        | <0.0005 |
| Triglyceride (mg/dL)        | 110.1±74.0                | 171.6±158.8    | male; 137.0±97.0; female; 73.5±43.5 | <0.05 |
| HDL-C (mg/dL)               | 64.3±16.4                 | 34.6±6.5       | male; 64.0±26.0; female; 75.5±27.5 | <0.0001 |
| LDL-C (mg/dL)               | 124.7±30.7                | 72.2±26.1      | < 139.0           | <0.0001 |

Data are presented as means ± SD. p-values were determined by unpaired Student's t-test.

*Reference values for the Japanese (launched at Jichi Medical University Hospital).

Abbreviations: BMI, body mass index; WBC, white blood cell; Hb, hemoglobin; Plt, platelet; FDP, fibrin/fibrinogen degradation products; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDH, lactate dehydrogenase; CPK, creatine phosphokinase; UN, urea nitrogen; UA, uric acid; NA, not available; NS, not significant.
Since DEX is an anti-inflammatory agent with inhibitory effects on the synthesis and signaling of IFNs,18-21 DEX could affect 25HC production. Therefore, the concentrations of 25HC/TC in the patients who did not receive DEX therapy were significantly higher than those of the 13 patients who underwent DEX therapy (Supplementary Fig. 3).

To determine which oxysterol is the most useful biomarker for discriminating COVID-19 from controls, we performed receiver operating characteristic (ROC) analysis (Supplementary Fig. 4). The areas under the curve (AUC) of the ROC curves were 0.73, 0.59, 0.68, 0.71, 0.61 and 0.82 for 25HC/TC, 4βHC/TC, 7αHC/TC, 7KC/TC, 24SHC/TC and 27HC/TC, respectively (Supplementary Table 2). The cutoff values were 8.05 ng/mg, 9.70 ng/mg, 44.9 ng/mg, 93.7 ng/mg, 32.3 ng/mg and 94.4 ng/mg for 25HC/TC, 4βHC/TC, 7αHC/TC, 7KC/TC, 24SHC/TC and 27HC/TC, respectively (Supplementary Table 2).

**Correlation between serum oxysterols and laboratory data in COVID-19 patients**

Using ELISA, we measured the serum concentrations of representative cytokines, such as interleukin 6 (IL-6) and IFN-γ, in COVID-19 patients. IL-6 is well recognized as a COVID-19 severity marker. Its inhibitors have been recommended for the treatment of acute respiratory distress syndrome (ARDS) associated with COVID-19 based on the concept of “cytokine storm”.26 IFN-γ is also a major cytokine produced by immune cells during viral infections. IL-6 and IFN-γ in COVID-19 patients at the time of discharge were significantly lower than those on admission (Supplementary Fig. 5); thus, IL-6 and IFN-γ may reflect the state of COVID-19.

To investigate whether serum oxysterol levels reflected the progression of COVID-19, we correlated serum oxysterols with some laboratory parameters that have been reported as COVID-19 severity markers, including IL-6 and IFN-γ (Fig. 2). Contrary to our expectations, most of the laboratory parameters, except for FDP, were not significantly correlated with 25HC levels. Similarly, other oxysterols were not significantly correlated with specific laboratory data (data not shown).

**Effects of LPS, IFNs, and DEX on CH25H expression in macrophages and hepatocytes**

To find out why 25HC was only moderately increased in COVID19 patients, HMDMs were treated with LPS and type I/II interferon and measured the expression of CH25H in macrophages. As shown in Fig. 3A, the expression of CH25H in HMDMs was strongly upregulated by LPS, IFN-α, and IFN-γ.

Subsequently, we investigated the effect of DEX, which was administered to most patients with severe COVID-19 in this study, on Ch25h expression in RAW cells. As shown in Fig. 3B, the expression of Ch25h in RAW cells was downregulated by DEX treatment. In addition, DEX suppressed Ch25h expression induced by LPS, IFN-α, and IFN-
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Figure 2  Correlation between serum oxysterols and laboratory data in COVID-19 patients. Oxysterol concentrations with TC correction correlated with laboratory parameters in COVID-19 patients on admission (n=17). The correlation coefficient r and p-values were determined using Spearman’s rank correlation test. *p < 0.05.

γ. Since the liver is also known to contribute to the production of oxysterols, we examined the effects of LPS, IFNs and DEX on the expression of the hepatic enzymes involved in the synthesis of oxysterols in HepG2 cells. LPS induced the expression of CH25H, which was reversed by DEX treatment. However, IFNs and DEX had no significant effects on CH25H expression (Supplementary Fig. 6A). Additionally, we assessed the expression of CYP7A1, Oxysterol 7α-hydroxylase (CYP7B1), and 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2), which regulate several oxysterols in the liver. As shown in Supplementary Fig. 6B, IFN-γ suppressed CYP7A1 gene expression; however, LPS, IFN-α, and DEX had no significant effects on the expression of CYP7A1, CYP7B1, and HSD11B2.

Discussion

The aim of the study was to determine whether COVID-19 affects serum concentrations of oxysterols and how it relates to the clinical picture. The serum concentrations of 25HC and 27HC were significantly increased; however, that of 7KC was significantly decreased in COVID-19 patients compared to the healthy controls (Supplementary Fig. 2). Since most serum oxysterols are associated with lipoproteins, the val-
ues were reanalyzed after normalizing for serum TC levels and were significantly decreased in COVID-19 patients, as reported previously.27 The results showed that the differences in 25HC/TC, 27HC/TC, and 7 KC/TC remained significant (Fig. 1). Serum 24SHC/TC levels were significantly higher in COVID-19 patients than in healthy controls.

In vitro studies revealed that the expression of the Ch25h gene is induced by LPS or IFNs in murine macrophages.6,7 In addition to murine macrophages, we confirmed that LPS or IFNs significantly increased the expression of CH25H in human monocyte-derived macrophages by approximately 100-fold (Fig. 3A), suggesting that bacterial and/or viral infection, which was associated with increased local or systemic concentrations of LPS or IFNs, might increase 25HC production by upregulating CH25H expression in macrophages.

The AUC of the ROC curve for 27HC was the largest, suggesting that 27HC is the most useful biomarker for COVID-19. However, it might be premature to mention the specificity at present, because we do not have biological explanations for the associations of serum 27HC with COVID-19.

Consistent with this hypothesis, the serum levels of 25HC were increased in COVID-19 patients. However, the magnitude of this increase was very modest; the 63% increase on the average was unimpressive compared to the 100-fold induction of CH25H mRNA in HMDM by LPS or IFNs in vitro. Moreover, most laboratory parameters known to be COVID-19 severity markers, such as lymphocyte number, LDH, and ferritin,27 were not significantly correlated with the serum levels of 25HC (Fig. 2). These considerations led to us proposing several explanations for this discrepancy.

First, pharmacotherapy during hospitalization may mitigate the induction of CH25H expression in macrophages. DEX is most likely the culprit because DEX inhibits the synthesis and signaling of IFN,18-21 which may, in turn, diminish CH25H gene expression and 25HC production by suppressing IFN production. The 25HC/TC ratio in patients receiving DEX therapy was significantly lower than in the untreated patients (Supplementary Fig. 3). DEX suppressed the Ch25h expression induced by LPS, IFN-α, and IFN-γ in RAW cells (Fig. 3B). In addition, DEX suppressed Ch25h expression, even in the absence of these inducers. Therefore, it is plausible that DEX inhibits CH25H expression by suppressing the production of IFNs and the expression of CH25H directly. Given the anti-viral activities of 25HC, it is possible that

Figure 3  Effects of LPS, IFNs, and DEX on CH25H mRNA expression in macrophages (A) RT-PCR analysis of CH25H mRNA expression of HMDMs treated with LPS (100 ng/mL) and IFNs (100 ng/mL) for 6 hours (n = 3). Data are presented as means ± SD. *p < 0.05, **p < 0.01, and ****p < 0.0001, as determined by unpaired Student’s t-test. (B) RT-PCR analysis of CH25H mRNA expression of RAW cells treated with LPS (100 ng/mL), IFNs (100 ng/mL) and DEX (1 μM) for 6 hours (n = 3). Data are presented as means ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, as determined by ANOVA.
DEX, which was used in suppressing the “cytokine storm” in COVID-19 patients, might negatively impact the clinical courses of COVID-19 by suppressing anti-viral 25HC production. The effects of DEX on the expression of IFNs and CH25H may account for the poor correlation between the serum levels of 25HC and IL-6/IFN-γ.

Second, even when the production of 25HC by alveolar macrophages is fully activated by infection with SARS-CoV-2 and subsequent overproduction of IFNs, its contribution to serum 25HC might be minor compared to the amount of 25HC produced by other organs, especially the liver, in a steady-state. Reportedly, CYP3A4,11 CYP27A1,13 CYP46A1,15 and autoxidation contribute to the production of 25HC.15 It is also noteworthy that the induction of CH25H expression by LPS is transient and may not be sustained long enough to substantially contribute to the high serum levels of 25HC.9,28 To resolve these issues, we needed to measure serum 25HC levels before DEX administration and compare their values with tissue levels of 25HC.

Recently, Marcello et al. reported that serum levels of 27HC, which was inhibitory to SARS-CoV-2, were significantly decreased in COVID-19 patients compared with the controls.13 He speculated that mitochondrial stress occurring at the systemic level could affect the activity of CYP27A1,29 thereby decreasing the serum levels of 27HC. This decreased serum levels of 27HC might be consistent with the findings that LPS potently downregulates the expression of CYP27A1 in the liver30 and macrophages.28

However, in our study, the serum levels of 27HC were significantly elevated in COVID-19 patients (Fig. 1). These contradictory results can be explained by the potential involvement of 27HC removal from the serum. CYP7B1 is known to hydroxylate 27HC at the 7α position to produce 5-cholesten-3β,7α,27-triol, which is further metabolized to chenodeoxycholic acid via an alternate pathway.31 CYP7B1 may be a more important determinant of serum 27HC than CYP27A1. Serum 27HC levels are markedly elevated in patients with hereditary spastic paraplegia type 5 (SPG5/HSP-CYP7B1), caused by biallelic variants of CYP7B1.32 Furthermore, 25HC was also revealed to be a substrate of CYP7B1 by reports showing increased serum levels of 25HC in mice33 and humans32 which are deficient in CYP7B1. Although LPS has been reported to significantly suppress the hepatic expression of CYP7B1 in mice,30 we were unable to show that LPS, IFNs, and DEX significantly changed CYP7B1 gene expression in HepG2 cells after 6 h of incubation (Supplementary Fig. 6B). Also, contrary to the results of Marcello et al.,7 serum 7KC levels were decreased in COVID-19 patients (Fig. 1). This finding was surprising because infections are associated with increased production of reactive oxygen species (ROS),34 which conceivably promote the production of 7KC.35 Besides autoxidation, 7KC is also produced by enzymes such as CYP7A136 and HSD11B2.37 Reportedly, LPS downregulates CYP7A1 and HSD11B2 in the liver. Although LPS, IFNs, and DEX did not change HSD11B2 gene expression during the 6 h incubation in HepG2 cells, IFN-γ suppressed CYP7A1 gene expression in HepG2 cells (Supplementary Fig. 6B). Therefore, it is conceivable that a decrease in the enzymatic production of 7KC overrides the increased non-enzymatic production of 7KC. Particularly, the concentrations of 25HC, 27HC, and 7KC in COVID-19 patients might be determined by changes in the production of oxysterols by macrophages and by changes in the expression of various hepatic CYPs. Further studies are required to clarify these discrepancies in 27HC and 7KC levels.

This study had several limitations. 1) Most of the study participants had severe COVID-19 necessitating treatment with DEX before blood sample collection; 2) the sample size was small; 3) the controls were not patients with other types of infection; and 4) serum oxysterol levels were measured only on admission.

In conclusion, we proved that the serum concentrations of 25HC and 27HC were increased; however, that of 7KC decreased in COVID-19 patients compared to healthy volunteers. These changes might reflect the induction of the expression of the genes responsible for their production, such as CH25H in macrophages, due to infection with SARS-COV2. The serum levels of 25HC may be used as a biomarker to estimate the severity of COVID-19.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the reported research.

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Authors’ contributions

Writing the manuscript: Takumi Asano, Tetsuji Wakabayashi, and Shun Ishibashi. Conceptualization and methodology: Daisuke Yamamuro, Manabu Takahashi, and Shun Ishibashi. Data acquisition: Takumi Asano, Tetsuji Wakabayashi, Yasuyuki Kondo, Kenta Okada, Yukiko Koga, Kiyonori Oka, Momoe Sakurai, Nagisa Sawayama, Kensuke Minami, Yuji Morisawa, Shuji Hatakeyama, and Masami Matsumura. Investigation and data analysis: Takumi Asano, Tetsuji Wakabayashi, and Daisuke Yamamuro.
References
1. Li J, Lai S, Gao GF, Shi W. The emergence, genomic diversity and global spread of SARS-CoV-2. Nature. 2021;600:408–418.
2. Zhao J, Chen J, Li M, Chen M, Sun C. Multifaceted functions of CH25H and 25HC to modulate the lipid metabolism, immune responses, and broadly antiviral activities. Viruses. 2020;12:727.
3. Li C, Deng YQ, Wang S, et al. 25-hydroxycholesterol protects host against Zika virus infection and its associated microcephaly in a mouse model. Immunity. 2017;46:446–456.
4. Liu SY, Aliyari R, Chikere K, et al. Interferon-inducible cholesterol-25-hydroxylase broadly inhibits viral entry by production of 25-hydroxycholesterol. Immunity. 2013;38:92–105.
5. Lembo D, Cagno V, Civra A, Poli G. Oxysterols: An emerging class of broad spectrum antiviral effectors. Mol Aspects Med. 2016;49:23–30.
6. Wang S, Li W, Hui H, et al. Cholesterol 25-hydroxylase inhibits SARS–CoV-2 and other coronaviruses by depleting membrane cholesterol. EMBO J. 2020;39:e106057.
7. Zang R, Case JB, Yutuc E, et al. Cholesterol 25-hydroxylase suppresses SARS-CoV-2 replication by blocking membrane fusion. Proc Natl Acad Sci U S A. 2020;117:32105–32113.
8. Cao Q, Liu Z, Xiong Y, Zhong Z, Ye Q. Multiple Roles of 25-Hydroxycholesterol in Lipid Metabolism, Antivirus Process, Inflammatory Response, and Cell Survival. Oxid Med Cell Longev. 2020;2020:8993305.
9. Diczfalussy U, Olofsson KE, Carlsson AM, et al. Marked upregulation of cholesterol 25-hydroxylase expression by lipopolysaccharide. J Lipid Res. 2009;50:2258–2264.
10. Park K, Scott AL. Cholesterol 25-hydroxylation production by dendritic cells and macrophages is regulated by type 1 interferons. J Leukoc Biol. 2010;88:1081–1087.
11. Honda A, Miyazaki T, Ikegami T, et al. Cholesterol 25-hydroxylation activity of CYP3A. J Lipid Res. 2011;52:1500–1516.
12. Li X, Pandak WM, Erickson SK, et al. Biosynthesis of the regulatory oxysterol, 5-cholesten-3beta,25-diol 3-sulfate, in hepatocytes. J Lipid Res. 2007;48:2587–2596.
13. Lund E, Bjorkhem I, Furster C, Wikvall K. 24-, 25- and 27-hydroxylation of cholesterol by a purified preparation of 27-hydroxylation from pig liver. Biochim Biophys Acta. 1993;1166:177–182.
14. Lund EG, Guilezyardo JM, Russell DW. cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain. Proc Natl Acad Sci U S A. 1999;96:7238–7243.
15. Diczfalussy U. On the formation and possible biological role of 25-hydroxycholesterol. Biochimie. 2013;95:455–460.
16. Su Z, Deng YQ, Zhou C, et al. 25-Hydroxycholesterol is a potent SARS-CoV-2 inhibitor. Cell Res. 2020;30:1043–1045.
17. Marcello A, Civra A, Milan Bonotto R, et al. The cholesterol metabolite 27-hydroxycholesterol inhibits SARS-CoV-2 and is markedly decreased in COVID-19 patients. Redox Biol. 2020;36:101682.
18. Gessani S, McCandless S, Baglioni C. The glucocorticoid dexamethasone inhibits synthesis of interferon by decreasing the level of its mRNA. Journal of Biological Chemistry. 1988;263:7454–7457.
19. Hu X, Li WP, Meng C, Ivashkov LB. Inhibition of IFN-γ signaling by glucocorticoids. J Immunol. 2003;170:4833–4839.
20. Flammer JR, Dobrovolna J, Kennedy MA, et al. The type 1 interferon signaling pathway is a target for glucocorticoid inhibition. Mol Cell Biol. 2010;30:4564–4574.
21. Bhattacharyya S, Zhao Y, Kay TW, Muglia LJ. Glucocorticoids target suppressor of cytokine signaling 1 (SOCS1) and type 1 interferons to regulate Toll-like receptor-induced STAT1 activation. Proc Natl Acad Sci U S A. 2011;108:9554–9559.
22. Nagaku M, Kadawaki T, Yotsuanyagi H, et al. The Japanese Medical Science Federation COVID-19 Expert Opinion English Version. JMA J. 2021;4:148–162.
23. Yamamuro D, Yamazaki H, Osuga JI, et al. Esterification of 4β-hydroxycholesterol and other oxysterols in human plasma occurs independently of LCAT. J Lipid Res. 2020;61:1287–1299.
24. Honda A, Yamashita K, Haru T, et al. Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS. J Lipid Res. 2009;50:350–357.
25. Wakabayashi T, Takahashi M, Yamamuro D, et al. Inflammamase activation aggravates cutaneous xanthomatosis and atherosclerosis in ACAT1 (Acyl-CoA cholesterol acyltransferase 1) deficiency in bone marrow, Arterioscler Thromb Vasc Biol. 2018;38:2576–2589.
26. Coomes EA, Haghbayani H. Interleukin-6 in Covid-19: A systematic review and meta-analysis. Rev Med Virol. 2020;30:1–9.
27. Feingold KR. The bidirectional link between HDL and COVID-19 infections. J Lipid Res. 2021;62:100067.
28. Preuss I, Ludwig MG, Baumgarten B, et al. Transcriptional regulation and functional characterization of the oxysterol/EBI2 system in primary human macrophages. Biochem Biophys Res Commun. 2014;446:663–668.
29. Bjorkhem I. Are side-chain oxidized oxysterols regulators also in vivo? J Lipid Res. 2009;50:S213–S218 Suppl.
30. Memon RA, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR. In vivo and in vitro regulation of sterol 27-hydroxylase in the liver during the acute phase response. Potential role of hepatocyte nuclear factor-1. J Biol Chem. 2001;276:30118–30126.
31. Russell DW. Fifty years of advances in bile acid synthesis and metabolism. J Lipid Res. 2009;50:S120–S125 Suppl.
32. Schule R, Siddique T, Deng HX, et al. Marked accumulation of 27-hydroxycholesterol in SPG5 patients with hereditary spastic paraparesis. J Lipid Res. 2010;51:819–823.
33. Li-Hawkins J, Lund EG, Turley SD, Russell DW. Disruption of the oxysterol 7α-alpha-hydroxylation gene in mice. J Biol Chem. 2000;275:16536–16542.
34. Taylor JP, Tse HM. The role of NADPH oxidases in infectious and inflammatory diseases. Redox Biol. 2021;48:102159.
35. Ghaziel I, Sassi K, Zarrour A, et al. 7-Ketosteroids: Effects on viral infections and hypothetical contribution in COVID-19. J Steroid Biochem Mol Biol. 2021;212:105939.
36. Shinkyo R, Xu L, Tallman KA, Cheng Q, Porter NA, Guengerich FP. Conversion of 7-dehydrocholesterol to 7-ketosteroid is catalyzed by human cytochrome P450 7A1 and occurs by direct oxidation without an epoxide intermediate. J Biol Chem. 2011;286:33021–33028.
37. Vejux A, Abed-Vieillard D, Hajii K, et al. 7-Ketosteroids and 7β-hydroxycholesterol: In vitro and animal models used to characterize their activities and to identify molecules preventing their toxicity. Biochem Pharmacol. 2020;173:113648.
38. Feingold KR, Spady DK, Pollock AS, Moser AH, Grunfeld C. Endo-toxin, TNF, and IL-1 decrease cholesterol 7α-hydroxylation mRNA levels and activity. J Lipid Res. 1996;37:223–228.
39. Fu L, Chen YH, Bo QL, et al. Lipopolysaccharide downregulates 11β-hydroxysteroid dehydrogenase 2 expression through Inhibiting peroxisome proliferator-activated receptor-γ in placental trophoblasts. J Immunol. 2019;203:1198–1207.