**Clinical Significance of Serum 7-Ketocholesterol Concentrations in the Progression of Coronary Atherosclerosis**

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**Aim:** 7-Ketocholesterol concentrations can be measured in a blood sample; however, the relationship between blood 7-ketocholesterol concentrations and atherosclerotic disease is not well-known. The aim of this study was to clarify the clinical significance of serum 7-ketocholesterol concentrations (s-7KCHO) in the progression of coronary atherosclerosis.

**Methods:** One hundred and thirty-nine subjects with coronary artery disease (CAD, subjects with stable angina pectoris or acute myocardial infarction) and 43 subjects with normal coronary arteries were enrolled in the study. s-7KCHO was measured using gas chromatography mass spectrometry.

**Results:** s-7KCHO was significantly higher in subjects with CAD than in those with normal coronary arteries (normal coronary artery: 19.0 ± 11.3 ng/mL, CAD: 32.4 ± 23.1 ng/mL, *p* < 0.01). Furthermore, patients with multiple vessel disease had significantly higher s-7KCHO than those with single vessel disease. Multivariate analysis revealed that s-7KCHO was an independent variable for CAD (*p* < 0.01). In CAD subjects, the presence of acute myocardial infarction, number of affected vessels, and high sensitive C-reactive protein concentrations strongly correlated with s-7KCHO (*p* < 0.01, < 0.05, < 0.05, respectively).

**Conclusion:** These results indicate that high s-7KCHO is closely associated with the progression of coronary atherosclerosis and inflammation.


**Key words:** Oxysterol, 7-ketocholesterol, Coronary angiography, Coronary artery disease, Risk factor

**Introduction**

Recent basic and clinical studies have illustrated that oxidative stress plays an important role in the progression of atherosclerosis. Oxidative stress leads to the oxidation of products in vivo and numerous oxidation products have been investigated and their significance examined in atherosclerotic disease. 7-Ketocholesterol is known to be a major component of the cholesterol oxidation product, oxysterols, and is found in high concentrations in atherosclerotic plaques, which contribute to the development of atherosclerosis. Thus, 7-ketocholesterol is considered an important target factor in the prevention of cardiovascular events, and reflecting the pathogenesis of atherosclerotic disease, therefore has clinical applications; however, the clinical significance of blood 7-ketocholesterol concentrations (7KCHO) is not fully understood, because it is difficult to analyze these concentrations accurately. In the present study, we established a system measuring for serum 7-ketocholesterol concentrations (s-7KCHO) using gas chromatography mass spectrometry and attempted to clarify the clinical significance of s-7KCHO in the progression of coronary atherosclerosis.

**Methods**

**Study Population**

One hundred and eighty-two subjects who underwent coronary angiography at Toho University Sakura Medical Center participated in this study. The study population consisted of 139 subjects with coro-
nary artery disease (CAD, subjects with stable angina pectoris or acute myocardial infarction) and 43 subjects with normal coronary arteries (NCA). Coronary risk factors, including s-7KCHO, were compared. All subjects gave their written informed consent and the study protocol was approved by the local ethics committee.

**Angiographical Study**

Coronary angiography was performed by transfemoral or transbrachial approaches using a standard technique. Two experienced angiographers, who were single blinded to the study, reviewed all coronary angiograms. The severity of coronary stenosis was assessed with a worst-view projection. The percentage of luminal narrowing was recorded according to the American Heart Associations reporting system. Significant stenotic lesion was defined as ≥75% diameter stenosis, and the extent of coronary atherosclerosis was classified by the number of vessels with significant stenotic lesions. Lesions in the left main trunk were not observed in the present study. Of the study population, 68 subjects suffered from acute myocardial infarction (AMI). All AMI subjects had a confirmed culprit lesion, which was total or subtotal occlusion by coronary angiography. Diagnosis of AMI was based on: (1) a clinical history of central chest pressure pain, or tightness for 30 min or more, (2) ST-segment elevation greater than 0.1 mV in at least one standard or precordial lead, and (3) a rise in the serum creatine kinase concentration to more than twice the normal laboratory value. All subjects with a normal coronary artery (NCA) underwent coronary angiography for the evaluation of chest pain and/or abnormal electrocardiograph, and NCA was defined as the absence of significant stenosis and spastic reaction, which was provoked by intracoronary administration of acetylcholine.

**Estimation of Coronary Risk Factors**

Age, sex, hypertension, diabetes mellitus, obesity, smoking, family history of coronary artery disease, serum lipid concentrations, high sensitive C-reactive protein concentrations (hs-CRP), and s-7KCHO were examined as risk factors for coronary artery disease. Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and/or under antihypertensive treatment. Diabetes mellitus was defined as fasting blood glucose level ≥126 mg/dL and/or a history of diabetes mellitus. Body mass index was calculated from weight (kg) divided by the square of the height (m²) and obesity was defined as a body mass index ≥25 kg/m². Smoking was defined as positive if there was a current or past history of cigarette smoking. The family history of CAD was considered positive if angina pectoris and/or myocardial infarction were present in grandparents, parents or siblings.

**Blood Sampling**

Blood samples were collected from the antecubital vein in the morning after 12 h fasting. A blood sample was collected before cardiac catheterization in subjects with stable angina pectoris or NCA. Blood samples were collected from AMI subjects who had suffered a coronary event one month after the initial event. Total cholesterol (TC) and triglyceride (TG) concentrations were measured enzymatically using a kit from Nippon Shoji (Osaka, Japan) and an autoanalyzer (Hitachi 7150; Hitachi Tokyo, Japan). High density lipoprotein cholesterol (HDL-C) concentrations were measured by the selective inhibition method (Daiichi Pure Chemicals, Tokyo). The concentrations of low density lipoprotein cholesterol (LDL-C) were calculated using Friedewald’s formula (total cholesterol–high density cholesterol–triglycerides/5). Plasma glucose concentrations were measured using the glucose oxidase method. hs-CRP concentrations were measured using high sensitivity latex-enhanced immunonephelometrics on a Behring II analyzer.

**Measurement of 7KCHO**

7KCHO was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). 24,25,25,26,26,26-d7-7Ketocholesterol (d7-7KCHO) was prepared by IsoTEC (Miamisburg, OH, USA), and used as an internal standard. Dried pyridine was purchased from Merck (Darmstadt, Germany). Serum blood samples were stored immediately at −80°C, and were used as samples for the s-7KCHO assay within 1 week. A 500 μL aliquot of serum was mixed with 50 μL internal standard stock solution (10 μg/mL d7-7KCHO dissolved in 1 mL toluene/ethyl acetate=1:1, v/v), and 3 mL diethyl ether/hexane (2:3, v/v) containing 0.01% BHT. The mixture was flushed with nitrogen gas and mixed in a rotary shaker for 30 min at room temperature. After centrifugation at 2,000 × g for 15 min, the organic phase was collected and dried under nitrogen gas. The residue was dissolved in 1 mL toluene/ethyl acetate (1:1, v/v), and applied to 3 mL “Diol” extraction columns (Bakerbond Spe; J.T. Baker Inc., Phillipsburg, NJ) that had been conditioned with the same solvent. After collection of the first eluted fraction under a mild vacuum, the columns were eluted with another 2 mL of the same solvent. Three millilitre of the eluent was dried under nitrogen gas, the residue was dis-
solved in 2 mL diethyl ether, and then 500 µL of 20% potassium hydroxide dissolved in methanol was added. After mixing in a rotary shaker for 3 h, the mixture was neutralized with 20% acetic acid. To separate the organic phase from the aqueous phase, 1 mL water was added and centrifuged. The organic phase was separated, added again to 1 mL water, and centrifuged. The pooled organic fractions were dried under nitrogen gas. Dried samples were derivatized with 200 µL o-methylhydroxylamine/hydrochloride dissolved in dried pyridine at 70°C for 2 h, and 100 µL N,O-bis (trimethyl-silyl) trifluoroacetamide (BSTFA) at 70°C for 2 h. Then, 1 µL aliquots were injected into a Varian GC/MS system consisting of a gas chromatograph (CP-3800), an ion trap mass spectrometer (Saturn-2000), and an auto-sampler (CP-8400). The whole instrument set was controlled by a computer. The column was a commercial product “WCOT Fused Silica (30 m × 0.25 mm i.d.) Coating CP-SIL 8 CB Low Bleed/MS” (Varian Inc., Palo Alto, CA, USA). Helium was used as the carrier gas at a flow rate of 0.8 mL/min. The injection temperature was set at 270°C (split ratio 1:4) and the initial column temperature at 60°C. The initial temperature was held for 1 min, and then increased at a rate of 20°C/min up to 280°C and 10°C/min up to 300°C. Thereafter, the temperature was held at 300°C for 10 min and was then increased again at a rate of 15°C/min until 330°C. The total running time was 31 min. The transfer line was maintained at 250°C and the ion source at 220°C. Electron ionization was performed by 70 eV ionized energy. The number of monitoring ions was 471 for 7KCHO and 478 for the internal standard (d7-7KCHO). In this assay system, the recovery test was 95%, intra-assay coefficients of variation were <5%, and the detection limit of the assay was 6.2 ng/mL.

Statistical Analysis

A commercially available statistical software program (Stat View-J 5.0; HULINKS Inc., Tokyo, Japan) was used for all statistical analyses. Data are expressed as the mean ± standard deviation. Between-group comparisons were performed using Student's t-test or the Mann-Whitney U test and the correlation coefficient was estimated by Spearman’s rank correlation analysis. Multivariate analysis was performed using multivariate logistic regression analysis or multiple regression analysis. P<0.05 was considered significant.

Results

Patient Characteristics

Patient characteristics are shown in Table 1. Age, the proportion of male subjects, diabetes mellitus and smoking were significantly higher in subjects with CAD than in those with NCA. Considering serum lipid concentrations, there were significantly higher TC, LDL-C and triglyceride concentrations in subjects with NCA than in those with CAD; however, the serum lipid data were similar in subjects without antihyperlipidemic treatment (data not shown). HDL-C concentrations were significantly lower in subjects with CAD than in those with NCA. hs-CRP concentrations were significantly higher in subjects with CAD than in those with NCA.

<table>
<thead>
<tr>
<th>Table 1. Baseline clinical characteristics</th>
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<tr>
<td></td>
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<tr>
<td>NCA (n=43)</td>
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<tr>
<td>CAD (n=139)</td>
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<td></td>
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<tr>
<td>Age (yrs)</td>
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<tr>
<td>Sex (male/female)</td>
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<tr>
<td>Hypertension</td>
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<tr>
<td>Diabetes mellitus</td>
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<tr>
<td>Obesity (BMI≥25)</td>
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<tr>
<td>Smoking</td>
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<tr>
<td>Family history of CAD</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
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<tr>
<td>HDL-cholesterol (mg/dL)</td>
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<tr>
<td>Antihyperlipidemic treatment</td>
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<tr>
<td>Statin use</td>
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<tr>
<td>High sensitive CRP (mg/L)</td>
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</table>

Data are expressed as the mean ± SD (t); %
*p<0.05 vs NCA, **p<0.01 vs NCA
NCA (n=43): Normal coronary artery, CAD (n=139): Coronary artery disease, BMI: body mass index, LDL: low-density lipoprotein, HDL: high-density lipoprotein, CRP: C reactive protein

Relationship between Coronary Atherosclerosis and 7KCHO

Comparisons of s-7KCHO between NCA and CAD subjects are shown in Fig.1. s-7KCHO was significantly higher in subjects with CAD than in those with NCA (p<0.01) (NCA: 19.0 ± 11.3 ng/mL, CAD: 32.4 ± 23.1 ng/mL). We also examined s-7KCHO in subjects not taking statins. s-7KCHO was significantly higher in subjects with CAD than in those with NCA (p<0.01) (NCA: 17.7 ± 9.6 ng/mL, CAD: 35.4 ± 25.4 ng/mL). The relationship between s-7KCHO and the number of affected vessels is shown in Fig.2. s-7KCHO was significantly higher in subjects with 2- or 3-vessel disease than in those with single vessel disease (p<0.05, p<0.05, respectively) (1-vessel dis-
ease: 28.0 ± 21.1 ng/mL, 2-vessel disease: 39.0 ± 25.6 ng/mL, 3-vessel disease: 41.3 ± 23.2 ng/mL). Comparisons of s-7KCHO between subjects with stable angina pectoris and those with AMI are shown in Fig. 3. s-7KCHO was significantly higher in subjects with AMI than in those with stable angina pectoris even though the number of affected vessels was similar in the two groups (p < 0.01) (stable angina pectoris: 26.1 ± 15.2 ng/mL, AMI: 39.1 ± 27.7 ng/mL).

Relationship between Coronary Risk Factors and 7KCHO

The correlation between serum parameters and s-7KCHO in CAD subjects is shown in Table 2. There were no significant correlations between TC or LDL cholesterol concentrations and s-7KCHO. Conversely, s-7KCHO had significant correlations with HDL-cholesterol and hs-CRP concentrations. Other coronary risk factors, such as age, sex, hypertension, diabetes mellitus, obesity, smoking, and a family history of CAD, were not related to s-7KCHO (data not shown). We also examined the relationship between statin use and s-7KCHO. Subjects taking statin use showed higher s-7KCHO than those not, although the difference was not significant (p = 0.09) (without statins: 28.9 ± 19.9 ng/mL, statins: 35.4 ± 25.7 ng/mL).

Multivariate Analysis

To investigate s-7KCHO as an independent cor-
Coronary risk factor, we performed multivariate logistic regression analysis for CAD (Table 3). s-7KCHO was selected as an independent variable for the presence of coronary artery disease (odds ratio, 1.06; 95% confidence interval, 1.02–1.09; \( p < 0.01 \)). Furthermore, we performed multiple regression analysis for s-7KCHO in CAD subjects (Table 4); the presence of AMI, number of affected vessels, and hs-CRP concentrations were independently associated with s-7KCHO (\( r = 3.9, 3.0, 2.9, p < 0.01, < 0.05, < 0.05 \), respectively).

**Discussion**

s-7KCHO was significantly higher in subjects with CAD than in those with NCA; furthermore, multiple regression analysis revealed that s-7KCHO was independent variable for the presence of CAD as a subordinate factor. The presence of AMI, number of affected vessels, and hs-CRP concentrations strongly correlated with s-7KCHO in CAD subjects.

**Significance of s-7KCHO in the Progression of Coronary Atherosclerosis**

7KCHO is excessive in advanced atherosclerotic plaques, and contributes to the development of atherosclerosis. Furthermore, 7KCHO causes apoptosis and inhibits the migration of smooth muscle cells. These findings suggest that the accumulation of 7KCHO in atherosclerotic lesions may decrease the number of cells and render atherosclerotic plaques unstable. Thus, 7KCHO is important not only in the progression of coronary atherosclerosis but is also the cause of plaque rupture, which is a major factor of AMI. In the present study, s-7KCHO reflected the severity of coronary atherosclerosis, estimated by coronary angiography; furthermore, s-7KCHO strongly correlated with the presence of AMI; therefore, s-7KCHO may reflect 7KCHO in the coronary artery plaque and is expected to be a predictor of AMI occurrence, which is strongly associated with mortality.

Hypercholesterolemia has been established as one of the most important coronary risk factors; however, the average serum TC or LDL-C concentration in subjects with CAD often appears to be within the norm range in the Japanese population. The present study also indicated that serum TC or LDL-C concentrations were not higher in CAD subjects than in NCA subjects. Furthermore, there was no relationship between serum TC or LDL-C concentrations and s-7KCHO. Another study also indicated that blood 7KCHO had no relationship with blood cholesterol concentrations in CAD subjects. Therefore, these results suggest that s-7KCHO is a different marker from blood cholesterol concentrations even though 7KCHO is an oxidation product of cholesterol. This allows us to predict coronary events which are not detectable by serum TC or LDL-C concentrations if we can measure s-7KCHO in the clinic. In a basic study, degenerative LDL, such as small LDL or glycated LDL, was shown to be easily oxidized, consequently promoting atherosclerotic lesion. Some clinical studies have emphasized an increase in degenerative LDL in CAD. 7KCHO may relate to oxidation products of degenerative LDL, however, the relationship is not fully understood; therefore, further studies are required to investigate the significance of s-7KCHO from the perspective of precise lipid profiles.

Among the serum lipid concentrations, only HDL-C had a significant relationship with s-7KCHO. A number of studies reported that low HDL-C concentrations were inversely related to the risk of cardio-

### Table 3. Results of multivariate logistic regression analysis for coronary artery disease

<table>
<thead>
<tr>
<th>Explanatory factor</th>
<th>OR (95% CI)</th>
<th>( p ) value</th>
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</thead>
<tbody>
<tr>
<td>s-7KCHO</td>
<td>1.06 (1.02–1.09)</td>
<td>( &lt; 0.01 )</td>
</tr>
<tr>
<td>Age</td>
<td>1.08 (1.03–1.13)</td>
<td>( &lt; 0.01 )</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>5.04 (1.81–13.98)</td>
<td>( &lt; 0.01 )</td>
</tr>
<tr>
<td>Diabetes</td>
<td>2.29 (0.85–6.13)</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>0.97 (0.94–1.01)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking</td>
<td>1.66 (0.62–4.42)</td>
<td>NS</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>1.10 (0.36–3.41)</td>
<td>NS</td>
</tr>
</tbody>
</table>

OR: odds ratio, CI: confidence interval, \( n = 182 \)
Abbreviations as in Table 1 and Fig. 1.

### Table 4. Results of multiple regression analysis for 7-ketocholesterol concentrations in subjects with coronary artery disease

<table>
<thead>
<tr>
<th>Explanatory factor</th>
<th>Standard regression coefficient</th>
<th>( r ) value</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMI</td>
<td>0.32</td>
<td>3.9</td>
<td>( &lt; 0.01 )</td>
</tr>
<tr>
<td>No of affected vessels</td>
<td>0.25</td>
<td>3.0</td>
<td>( &lt; 0.05 )</td>
</tr>
<tr>
<td>hs-CRP</td>
<td>0.24</td>
<td>2.9</td>
<td>( &lt; 0.05 )</td>
</tr>
</tbody>
</table>

Subordinate factor

\[ s-7KCHO \ (\text{actual value}) \]

\[ R^2 = 0.27, F = 3.8, p < 0.0001, \ (n = 139) \]
Abbreviations as in Table 1, Fig. 1. and Fig. 3.
vascular disease\textsuperscript{38, 39} and the present study may indicate that HDL retards the progression of coronary atherosclerosis by decreasing s-7KCHO in the coronary vessel wall or blood flow. The relationship between HDL-C concentrations and s-7KCHO in coronary atherosclerosis is not fully understood. Recently, however, Terasaka et al. reported that HDL exerted a protective effect against apoptosis induced by 7KCHO using ATP-binding cassette transporter ABCG1\textsuperscript{10}. The ATP-binding cassette transporter ABCG1 was recently shown to promote the efflux of cholesterol from macrophage to HDL and reverse the relationship between HDL-C concentrations and s-7KCHO. This may be partly explained by the role of ATP-binding cassette transporter ABCG1.

Relation between Concentration of s-7KCHO and Inflammation

In the present study, there was a significant correlation between s-7KCHO and hs-CRP concentrations in CAD subjects. Furthermore, these two factors had a significant association after adjustment of related factors. Thus, this result suggests that s-7KCHO is closely associated with inflammation in coronary atherosclerosis. Recent clinical investigations showed that inflammation plays an important role in the progression of atherosclerosis\textsuperscript{37, 42} and epidemiological studies clarified the link between high levels of CRP concentration and cardiovascular events\textsuperscript{38, 41}. Paul et al. showed that CRP is present in the human arterial intima of atherosclerotic lesions by histological examination\textsuperscript{45}; furthermore, Kobayashi et al. reported that, using a sample from directional coronary atherectomy, the expression of CRP was colocalized with p22phox, and CRP directly induced p22phox expression, generating reactive oxygen species in cultured smooth muscle cells\textsuperscript{40}. Therefore, CRP may promote the production of 7-KCHO in coronary artery plaques, consequently increasing s-7KCHO, however, some reports have indicated that oxysterol influences proinflammatory properties\textsuperscript{43-51}. Joffre et al. reported that 7KCHO enhanced interleukin-8 gene expression using porcine retinal pigment epithelial cells\textsuperscript{30}. Interleukin-8 is a proinflammatory and chemotactic cytokine which might play a crucial role in the recruitment of monocytes and T lymphocytes into the arterial subendothelial space, consequently promoting atherosclerotic lesions\textsuperscript{52}. Furthermore, interleukin-8 could have a potential atherogenic role by inhibiting the local tissue inhibitor of metalloproteinase-1 expression, thereby leading to an imbalance between matrix metalloproteinases and metalloproteinase-1 at focal sites of atherosclerotic plaque, and local extracellular degradation, causing the rupture of atheromatous plaques\textsuperscript{53}. Thus, 7KCHO and inflammation in coronary atherosclerosis are closely associated and may promote vulnerable plaque formation.

Limitations

Since this was a cross sectional study and the study population was small, a further study involving a larger number of subjects is needed to evaluate high s-7KCHO as a coronary risk factor. Simple and reliable methods to measure blood 7KCHO concentrations are therefore needed and the examination of many clinical studies investigating the significance of s-7KCHO in diagnosis and treatment is required.

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