Hypercholesterolemia in Minipigs Impairs Left Ventricular Response to Stress
Association With Decreased Coronary Flow Reserve and Reduced Capillary Density

Gregor Theilmeier, MD*; Peter Verhamme, MD*; Steven Dymarkowski, MD; Heike Beck, MD; Hilde Bernar; Marleen Lox; Stefan Janssens, MD, PhD; Marie-Christine Herregods, MD, PhD; Erik Verbeken, MD, PhD; Désiré Collen, MD, PhD; Karl Plate, MD; Willem Flameng, MD, PhD; Paul Holvoet, PhD

Background—Hypercholesterolemia induces functional and structural changes of the microvasculature and reduces coronary flow reserve in humans and experimental animals. The effect of hypercholesterolemia on left ventricular (LV) function in the absence of coronary stenosis is, however, unknown. Our objective was therefore to assess the effect of hypercholesterolemia and cholesterol withdrawal on LV function in the presence of advanced coronary plaques that do not cause stenosis.

Methods and Results—Twenty-eight minipigs on cholesterol diet for 34 weeks and 16 control pigs were studied. Seven hypercholesterolemic pigs were withdrawn from the diet for 26 weeks. LV function was assessed with cine-MRI, myocardial blood flow with colored microspheres, and capillary density with immunohistochemistry, and microvascular endothelial cell apoptosis with terminal dUTP nick-end labeling staining. Hypercholesterolemia (17±8 versus 268±150 versus 12±10 mg/dL LDL cholesterol, control versus hypercholesterolemic versus cholesterol withdrawal; P<0.001) induced atherosclerosis but not stenosis in the left coronary artery. Baseline cardiac output, ejection fraction, and stroke volume were similar in control and hypercholesterolemic pigs. In dobutamine stress test, cardiac output (P<0.05) and stroke volume (P<0.01) were lower in hypercholesterolemic pigs compared with controls. The impaired response to dobutamine was reversible by dietary cholesterol withdrawal. Hypercholesterolemia reduced endomyocardial coronary flow reserve (P<0.01) and capillary density (P<0.05) and induced capillary endothelial cell apoptosis. Hypercholesterolemic pigs failed to reduce vascular resistance in response to increased LV workload and pharmacological vasodilation.

Conclusion—LDL hypercholesterolemia in minipigs impaired LV response to dobutamine stress in the absence of coronary stenosis. (Circulation. 2002;106:1140-1146.)

Key Words: arteriosclerosis ■ cardiac output ■ capillaries ■ microspheres ■ hypercholesterolemia

Coronary artery disease and ischemic cardiomyopathy are major causes of morbidity and mortality worldwide. Hypercholesterolemia is a major risk factor for coronary artery disease. Deterioration of left ventricular (LV) function has mainly been attributed to coronary insufficiency secondary to formation of atherosclerotic lesions in epicardial coronary arteries that reduce blood flow at rest or in response to increased demands.1 Hypercholesterolemia also reduces coronary flow reserve and induces microvascular dysfunction2–4 that may contribute to myocardial ischemia and LV dysfunction. Patients with syndrome X have myocardial ischemia without stenosing coronary artery lesions on the basis of microvascular endothelial dysfunction.5,6 Microvascular dysfunction is also present in patients with dilated cardiomyopathy.7

To date, there have been no reports on the effects of hypercholesterolemia-induced microvascular changes on LV function in the absence of epicardial coronary stenosis. We therefore examined the effect of hypercholesterolemia on LV performance in diet-induced hypercholesterolemia and after
dietary cholesterol lowering in minipigs. We also studied the relation of LV function after dobutamine stress testing with endo- and epicardial coronary flow reserve, LV capillary density, and atherosclerosis in the left anterior descending coronary artery (LAD).

Methods

Animal Procedures

The local institutional review board approved all animal procedures. Minipigs\(^8\) were obtained by crossbreeding Gottinger and Yucatan minipigs (Charles River Laboratories, Lyon, France). Twenty-eight minipigs were placed on a 4\%-cholesterol diet for 34±5.7 (mean±SD) weeks. After that period, 7 hypercholesterolemic pigs were continued on a regular low-cholesterol chow for 26±1 weeks to study the effect of dietary cholesterol lowering. Sixteen age-matched pigs were used as controls.

Blood Sampling

Peripheral venous blood was drawn from an ear vein. Total cholesterol, HDL cholesterol, and triglyceride levels were measured in the university hospital routine laboratory. LDL cholesterol levels were calculated with the Friedewald formula. Circulating oxidized LDL (ox-LDL) was measured as previously described.\(^9\) Blood viscosity was measured in 8 additional control and 8 hypercholesterolemic pigs with an LVDV-II+ Brookfield viscosimeter (Analys) with UL-adapter at 37°C.

MRI

MRI was performed on a 1.5-T imager (Siemens Magnetom Vision). According to stress level, cardiac MRI was acquired in 8 short-axis views and 2 horizontal long-axis views. A segmented k-space gradient-echo sequence (TR/TE/\(t\) 4.8/60 ms/20\(^{°}\); matrix, 110×256; field of view, 196×250 mm; slice thickness, 6 mm) was used. The amount of phases acquired per R-R interval was adapted to the effective heart rate of the animal, so that both systole and the largest amount of phases acquired per R-R interval was adapted to the field of view, 196\(^{°}\). Ventricular pressures and heart rate (HR) were recorded. Dobutamine (18\(\mu\)g) were injected into the LV. Simultaneously arterial and coronary sinus views and 2 horizontal long-axis views. A segmented k-space gradient-echo sequence (TR/TE/\(t\) 20/15 ms/8\(^{°}\); matrix, 110×256; field of view, 196×250 mm; slice thickness, 6 mm) was used. The amount of phases acquired per R-R interval was adapted to the effective heart rate of the animal, so that both systole and the largest part of diastole were covered. Cardiac MRI was obtained during a regular low-cholesterol chow for 26±1 weeks to study the effect of dietary cholesterol lowering. Sixteen age-matched pigs were used as controls.

Coronary Artery Instrumentation

Coronary arteries were instrumented in deep propofol anesthesia as described before,\(^11\) and intravascular ultrasound (IVUS) loops of the proximal LAD were recorded to exclude significant epicardial coronary stenosis (IVUS Visions Five-64FX, Endosonics). Lumen size was determined offline on calibrated IVUS-loops with the built-in image analysis tool of the IVUS device. Coronary angiograms were obtained from all hypercholesterolemic pigs.

Myocardial Blood Flow and Resistance

LV myocardial blood flow was determined by injecting 15-\(\mu\)m colored microspheres (CMs; Dye-Trak, Triton Technology) as described elsewhere.\(^12\) Hemodynamic parameters were allowed to stabilize for 10 minutes before 5 mL of thoroughly mixed CMs (18x10\(^{10}\)) were injected into the LV. Simultaneously arterial and ventricular pressures and heart rate (HR) were recorded. Dobutamine was started and titrated in 2.5 \(\mu\)g · kg\(^{-1}\) · min\(^{-1}\) steps to increase HR by 20% when a second dose of CMs was injected. Hemodynamics was recorded and dobutamine was discontinued; 0.8 mg/kg body weight of dipyridamole was injected into the LV, and a third dose of CMs was injected 5 minutes thereafter. The heart was removed, fixed, and divided into 5 slices of equal thickness. The central slice was used for spectrophotometric analysis of CM distribution. Coronary flow reserve was defined as the ratio of myocardial flow after dipyridamole to resting blood flow. Coronary vascular resistance was calculated based on flow and mean arterial pressure.

Histology and Immunohistochemistry

The LAD was excised after perfusion fixation with paraformaldehyde and divided into rings for histological processing. Blocks of myocardium from the anterior free wall were likewise excised from the hearts and cryoembedded for immunohistochemistry and terminal deoxyuridine nick-end labeling (TUNEL)-staining. Coronary lesions were determined morphometrically by delineating the external elastic lamina, the internal elastic lamina, and the lumen. Transmural myocardial sections were stained with biotinylated Blandeira simplicifolia isolectin B4 (Vector Laboratories) and developed with streptavidin-peroxidase conjugate to stain endothelial cells. Capillary density was assessed histomorphometrically on a Quantimet 600 (Leica) by manual delineation of capillaries in 8 high-power fields in the endomyocardial and 8 high-power fields in the epicardiovascular layers of the free anterior LV wall, and a mean was established. Care was taken to only analyze areas with transverse capillary profiles.

Endothelial Cell Apoptosis

TUNEL assay was performed according to the manufacturer protocols (Intergen). Endothelial cells were labeled with a rabbit polyclonal anti-human von Willebrand Factor antibody (DAKO). Biotinylated goat-anti-rabbit antibody was used as secondary antibody. Omission of the primary antibody served as control. The peroxidase reaction was visualized with 3-amo-9-ethylcarbazole (Vector Laboratories) and 0.006% H\(_2\)O\(_2\). The presence of apoptotic endothelial cells was semi-quantitatively scored on 5 randomly chosen high-magnification fields (200\(^{×}\)) of 4 different sections of each animal. Apoptotic endothelial cells were considered present if \(≥3\) TUNEL-positive endothelial cells per high power field were counted.

Statistics

Groups were compared by nonparametric ANOVA followed by Dunnett’s test (SPSS, version 10). Paired nonparametric testing compared hemodynamics and myocardial function at resting conditions and at stress testing. Correlations were determined by Spearman test. Multiple regression analysis was performed to determine the relation of cardiac output (CO) with LDL cholesterol, coronary flow reserve, capillary density, and atherosclerosis in LAD. Values of \(P<0.05\) were considered statistically significant.

Results

Lipids and Atherosclerosis

The cholesterol-rich diet induced LDL hypercholesterolemia (Table 1). Levels of oxidized LDL (ox-LDL) were higher in hypercholesterolemic pigs. Triglyceride levels were similar in control and hypercholesterolemic pigs (Table 1). Blood viscosity was 26% higher in hypercholesterolemic versus control pigs (5.4±0.63 versus 4.7±0.28 mPa\(\times\)s; \(P<0.01\)). Cholesterol withdrawal resulted in a decrease of LDL cholesterol and ox-LDL (Table 1).

Mean intimal area in the proximal LAD was 0.81±0.17 mm\(^2\) in hypercholesterolemic compared with 0.15±0.03 mm\(^2\) in control pigs (\(n=13\) and 16, \(P<0.01\)). Lumen area of the LAD, assessed by IVUS, was 9.2±0.6 mm\(^2\) in control and 9.6±0.4 mm\(^2\) in hypercholesterolemic pigs (\(P=NS\)). Coronary angigrams did not reveal significant stenosis. Cholesterol withdrawal did not significantly change the available lumen area of the LAD determined by IVUS (10.4±1.4 versus 10.1±1.1 mm\(^2\), hypercholesterolemic versus cholesterol withdrawal; \(n=6, P=NS\)). Mean intimal area in pigs after dietary cholesterol withdrawal was not different from that in...

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hypercholesterolemic pigs (1.14±0.45 versus 0.81±0.17 mm2; n=7 and n=17).

**LV Function**
Under resting conditions, HR, CO, EF, SV, EDV, and ESV were similar in control and hypercholesterolemic pigs (Table 2). To increase HR by 20%, higher dobutamine doses were required in the hypercholesterolemic and cholesterol withdrawal groups compared with controls (ANOVA P<0.05) (Table 2). Dobutamine stress test significantly increased CO in controls but not in hypercholesterolemic pigs (37±28% versus 3.3±27%, P<0.05) (Table 2). In hypercholesterolemic pigs, EF (P<0.05) and SV (P<0.05) decreased after dobutamine stress test (Table 2). There were no differences between hypercholesterolemic and cholesterol-lowering pigs before cholesterol withdrawal was initiated, both at rest and after dobutamine stress. In cholesterol withdrawal pigs, resting LV function was similar before and after cholesterol withdrawal (data not shown). After 6 months of cholesterol withdrawal, CO, EF, and SV in response to dobutamine were similar those of control pigs (Table 2). After dobutamine stress test, SV was decreased in hypercholesterolemic pigs and increased in controls and pigs after cholesterol withdrawal (ANOVA P<0.05). The increase in CO was higher in controls than in hypercholesterolemic pigs. After dietary cholesterol lowering, increase in CO was not different from that in control pigs (ANOVA P<0.05) (Figure 1).

**Coronary Flow**
Blood pressure tended to be lower in hypercholesterolemic pigs at rest, after dobutamine and after dipyridamole (Table 3). Resting endo- and epimyocardial blood flow tended to be higher in hypercholesterolemic versus control pigs (Table 3). Endomyocardial flow was significantly higher than epimyo-cardial flow in control pigs (P<0.05) but not in hypercholesterolemic pigs (Table 3). The endomyocardial-to-epimyocardial flow ratio was significantly higher in control pigs than in hypercholesterolemic pigs (P<0.05) (Figure 2). Coronary flow reserve was reduced in the endomyocardium of hypercholesterolemic pigs (P<0.01) (Figure 2). Dobutamine increased endo- and epimyocardial flow to 175% and 168%, respectively, of flow under resting conditions (P<0.01) in control pigs compared with 121% and 118% (P=NS), respectively, in hypercholesterolemic pigs. Dipyridamole caused a redistribution of blood flow away from the subendocardium in hypercholesterolemic pigs but not in control pigs (endomyocardial/epimyocardial flow ratio, 0.73±0.086 and 1.08±0.055, respectively; P<0.05) (Figure 2).

### TABLE 1. Lipid Values

<table>
<thead>
<tr>
<th></th>
<th>Control (n=16)</th>
<th>Hypercholesterolemic (n=19)</th>
<th>Cholesterol Withdrawal (n=7)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>LDL cholesterol</td>
<td>17±8</td>
<td>268±150</td>
<td>12±10</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>29±9</td>
<td>81±19</td>
<td>27±5</td>
<td>&lt;0.001</td>
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<tr>
<td>Triglycerides</td>
<td>65±36</td>
<td>49±27</td>
<td>59±46</td>
<td>NS</td>
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<tr>
<td>0x-LDL</td>
<td>0.73±0.15</td>
<td>2.67±1.29</td>
<td>0.85±0.11</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are mean±SD (mg/dL). P values were determined by nonparametric ANOVA.

### TABLE 2. Left Ventricular Function Measured by Cardiac Magnetic Resonance Imaging

<table>
<thead>
<tr>
<th></th>
<th>Control (A) (n=9)</th>
<th>Hypercholesterolemic (B) (n=18)</th>
<th>Cholesterol Withdrawal (C) (n=5)</th>
<th>ANOVA P</th>
<th>A vs B</th>
<th>A vs C</th>
<th>B vs C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resting conditions</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>HR, bpm</td>
<td>86±6.6</td>
<td>84±1.8</td>
<td>79±5.7</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CO, mL</td>
<td>3287±228</td>
<td>3350±245</td>
<td>2953±152</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EF, %</td>
<td>54±1.7</td>
<td>58±1.65</td>
<td>56±3.2</td>
<td>NS</td>
<td></td>
<td></td>
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<tr>
<td>SV, mL</td>
<td>38±1.6</td>
<td>39±1.66</td>
<td>38±2.2</td>
<td>NS</td>
<td></td>
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<tr>
<td>EDV, mL</td>
<td>70±2.0</td>
<td>69±2.0</td>
<td>67±4.7</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESV, mL</td>
<td>32±1.4</td>
<td>29±2.54</td>
<td>26±2.1</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Stress test (HR +20%)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Dobutamine dose</td>
<td>5.8±0.91</td>
<td>12±6.7</td>
<td>10±1.2</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>HR</td>
<td>109±24†</td>
<td>108±26‡</td>
<td>95±9.0</td>
<td>NS</td>
<td></td>
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<tr>
<td>CO</td>
<td>4441±1128†</td>
<td>3361±1011</td>
<td>3998±348</td>
<td>*</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>EF</td>
<td>59±11</td>
<td>51±11§</td>
<td>60±6.1</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV</td>
<td>41±7.22†</td>
<td>31±6.2†</td>
<td>42±4.7</td>
<td>*</td>
<td>¶</td>
<td>NS</td>
<td>*</td>
</tr>
<tr>
<td>EDV</td>
<td>69±4.6</td>
<td>62±9.9</td>
<td>71±6.7</td>
<td>*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ESV</td>
<td>28±9.0</td>
<td>31±7.8</td>
<td>28±5.9</td>
<td>NS</td>
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</table>

Data are mean±SD. P values determined by nonparametric ANOVA followed by Dunnett’s test or by paired nonparametric testing.

*P<0.05, †P<0.01, control vs hypercholesterolemic vs cholesterol withdrawal.
¶P<0.01, ‡P<0.001, §P<0.05, stress test vs resting conditions.
Dobutamine, to increase HR by 20%, and dipyridamole decreased mean endomyocardial and epimyocardial resistance in control pigs ($P<0.001$ for both) but failed to do so in hypercholesterolemic pigs (Figure 3).

**Capillary Density and Endothelial Cell Apoptosis**

Endo- and epimyocardial capillary densities were not different. Mean LV capillary density was smaller in hypercholesterolemic pigs compared with control pigs ($P<0.05$) (Figure 4). Oil red O staining of adjacent sections revealed single small plaques in branching points of precapillary arterioles but no disseminated microvascular coronary artery disease or overt lipid deposition in the myocytes themselves. Hematoxylin/eosin staining demonstrated no gross abnormalities of the examined myocardium. TUNEL staining scored semiquantitatively revealed microvascular endothelial cell apoptosis in the myocardium of 6 out of 13 hypercholesterolemic pigs compared with only 1 out of 8 of control pigs.

**CO, Myocardial Flow, and Capillary Density**

Figure 5 demonstrates that the change in CO after dobutamine stress testing correlated with endomyocardial coronary flow reserve and with capillary density. The change in CO after dobutamine stress did not correlate with the mean intimal area in the proximal LAD (Figure 5). Multiple regression analysis—using a model containing the change in CO, LDL cholesterol, coronary flow reserve, capillary density, and plaque area in the LAD—showed that coronary flow reserve predicted the change in CO after stress test ($R^2=0.42; P=0.04$).

**Discussion**

The main findings of the present study are that LDL hypercholesterolemia in minipigs is associated with impaired LV performance in response to inotropic stress. Cholesterol withdrawal restored LV performance in dobutamine stress testing. The atherogenic diet induced atherosclerosis in the LAD as reported before8; however, coronary stenosis in the epicardial arteries was not detected, excluding major coronary stenosis as the cause for LV dysfunction. Microvascular dysfunction was evidenced by reduced endomyocardial cor-

<table>
<thead>
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<th>TABLE 3. Blood Pressure and Coronary Flow</th>
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<tr>
<td>Control (n=10)</td>
</tr>
<tr>
<td>MAP</td>
</tr>
<tr>
<td>Endo flow</td>
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<tr>
<td>Epi flow</td>
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<tr>
<td>Hypercholesterolemic (n=14)</td>
</tr>
<tr>
<td>MAP</td>
</tr>
<tr>
<td>Endo flow</td>
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<tr>
<td>Epi flow</td>
</tr>
</tbody>
</table>

Data are mean±SD. MAP indicates mean arterial pressure; endo, endomyocardial; and epi, epimyocardial. Flow is expressed as blood flow per myocardial tissue (mL/g).

*P<0.01, †P<0.05, §P<0.001; resting vs dobutamine vs dipyridamole.
‡P<0.05, endo versus epi.
onary flow reserve, failure to decrease resistance in response to increased workload or pharmacological vasodilatation, disturbed transmural flow distribution, and reduced left capillary density. Thus hypercholesterolemia-induced microvascular changes were linked to LV dysfunction in the absence of stenosing coronary artery disease.

In hypercholesterolemic pigs, vascular dysfunction, as has been described in numerous clinical studies, was demonstrated by reduced coronary flow reserve and disturbed flow regulation. Dipyridamole caused a distribution of transmural flow away from the endomyocardium. This intracoronary steal effect occurred even in the absence of epicardial stenosis and may have affected LV function, which is strongly dependent on endomyocardial flow. Vascular resistance did not decrease in response to increased oxygen demand owing to dobutamine in hypercholesterolemic pigs, suggesting that endothelial dysfunction was in part responsible for impaired LV function with increased demand. The response of the LV to dobutamine stress testing correlated with indices of vascular dysfunction such as coronary flow reserve in the endomyocardium and myocardial capillary density, but not with the degree of epicardial atherosclerosis. Reduced capillary density evidenced also structural alterations in the microvasculature. The reduced capillary density in hypercholesterolemic pigs may reflect an imbalance between capillary loss because of endothelial cell apoptosis and capillary replacement. We have demonstrated microvascular endothelial cell apoptosis, whereas others have demonstrated impaired angiogenesis. Oxidized LDL induces endothelial cell apoptosis in vitro. In humans, coronary flow reserve inversely correlated with levels of autoantibody titer against oxidized LDL. Circulating levels of oxidized LDL were increased in hypercholesterolemic pigs and returned to baseline after lipid lowering. Hypercholesterolemia also reduces circulating endothelial cell progenitor cells, whereas lipid-lowering agents were found to restore the number of endothelial progenitor cells that could contribute to the neocapil-
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Hypercholesterolemia and LV Dysfunction

Theilmeier was a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft and is currently a member of the Interdisciplinary Center for Clinical Research Münster, “IZKF-The chronic disease” University of Münster, Germany. Dr Verhammer is a doctoral fellow of the FWO, Vlaanderen. We thank M. Landeloos and V. Leunens for excellent technical assistance.

References


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