Heart Disease in Children

Early Increase of Oxidative Stress and Soluble CD40L in Children With Hypercholesterolemia

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Objectives
The aim of the study was to analyze the behavior of oxidative stress and its interplay with CD40L, a protein that is implicated in atherosclerosis, in hypercholesterolemic children.

Background
Oxidative stress has been suggested to play a major role in premature atherosclerosis.

Methods
Forty-one children with hypercholesterolemia (mean age 9.28 ± 0.5 years) and 40 children with normocholesterolemia (mean age 9.02 ± 0.69 years) were matched for gender and age. Within each group, children were classified as having or not having a family history of cardiovascular disease. Serum levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative stress, and plasma levels of soluble CD40L (sCD40L) were measured in each child. In a subgroup of children with high (n = 8) or normal (n = 8) levels of serum cholesterol, platelet p38 mitogen-activated protein (MAP) kinase phosphorylation, a protein involved in the activation of nicotinamide adenine dinucleotide phosphate oxidase, was determined.

Results
Children with hypercholesterolemia had higher values of 8-OHdG and sCD40L compared with control subjects (0.55 ± 0.06 ng/ml vs. 0.21 ± 0.02 ng/ml, p < 0.001 and 0.55 ± 0.04 ng/ml vs. 0.19 ± 0.03 ng/ml, p < 0.001, respectively). A significant correlation between 8-OHdG and sCD40L was observed in children with high (r = 0.676, p < 0.001) or normal (r = 0.878, p < 0.001) levels of cholesterol. Children with a family history of cardiovascular disease tended to have higher values of 8-OHdG and sCD40L, but the difference was not significant. Analysis of platelet p38 MAP kinase showed that it was phosphorylated more in children with hypercholesterolemia compared with control subjects (36.8 ± 5.8 AU vs. 8.0 ± 4.5 AU, p < 0.001 respectively).

Conclusions
Children with hypercholesterolemia have an early increase of oxidative stress that may be responsible for upregulation of CD40L and potentially predispose to premature atherosclerosis. (J Am Coll Cardiol 2007;49: 1974–81) © 2007 by the American College of Cardiology Foundation

Atherosclerosis is a process that is already detectable in children with classic risk factors for atherosclerosis. Autopsy studies performed in children or youths with established risk factors demonstrated a positive association with the presence and extent of atherosclerotic lesions in the aorta and coronary arteries (1–3). Among the classic risk factors for atherosclerosis, total cholesterol (TC) seems to be a major determinant of early atherosclerosis. Thus, autopsy studies documented an association between TC and low-density lipoprotein (LDL) cholesterol with the extent and severity of atherosclerosis in infants, children, and adolescents (1–3).

Early increase of cholesterol is also relevant in the progression of atherosclerosis in adults; thus, measurement of LDL in childhood predicts carotid intima-media thickness in young adults (4).

Uptake of oxidized LDL by macrophages via scavenger receptors is believed to represent the early phase of atherosclerotic lesion (5). The key role of oxidative stress in LDL uptake by macrophages has been documented by demonstrating lower LDL uptake by human macrophages upon antioxidant supplementation (6). Histologic examinations have shown that macrophage infiltration in the coronary artery occurs in infancy, suggesting that oxidative stress with ensuing LDL modification is an early phenomenon associated with the onset of atherosclerosis (1,7,8). Using several markers of oxidative stress, including isoprostanes and 8-hydroxy-2′-deoxyguanosine (8-OHdG), an enhanced oxidative stress has been observed in adults with hypercholesterolemia (9,10). To our knowledge, there is only 1 report.
that documented enhanced oxidative stress in children with hypercholesterolemia and its decrease upon pravastatin treatment, indicating that cholesterol could be implicated in enhancing oxidative stress (11). To further explore whether oxidative stress is an early phenomenon influencing inflammatory processes related to atherosclerotic disease, we analyzed the relationship between 8-OHdG serum levels and plasma levels of soluble CD40L (sCD40L), an inflammatory protein involved in the onset and progression of atherosclerotic disease (12), in a population of children affected by hypercholesterolemia. Also, we examined whether a family history of cardiovascular disease, which is associated with early signs of subclinical atherosclerosis (13), influences oxidative stress and sCD40L in this clinical setting. Finally, we examined whether platelets from children with hypercholesterolemia have intra-cellular changes potentially accounting for enhanced oxidative stress; for this purpose, we measured platelet p38 mitogen-activated protein (MAP) kinase, a protein that is known to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (14), in a subgroup of children with hypercholesterolemia. We report that enhanced oxidative stress is detectable early in children with hypercholesterolemia and may contribute to the up-regulation of CD40L.

**Materials and Methods**

**Subjects and family classification.** Forty-one index children (ages 2 to 18 years) were recruited through a screening program of dyslipidemias in childhood. They were referred to the Lipid Clinic Research, Dipartimento di Pediatria, Università di Roma “La Sapienza,” Rome, Italy, to determine the presence of abnormal lipid values detected during an occasional laboratory test. The control group comprised 40 normocholesterolemic children matched for age and gender. The phenotype of the hypercholesterolemic children was classified as family hypercholesterolemia (n = 9) on the basis of the presence of a first-degree relative with hypercholesterolemia (TC >95th age- and gender-specific percentile); family combined hyperlipidemia (n = 13), defined as a presence of a first-degree relative with high triglycerides and/or high TC (TC >95th age- and gender-specific percentile); polygenic hypercholesterolemia on the basis of the presence of TC >95th age- and gender-specific percentile and without clear family transmission (n = 19) (15).

Exclusion criteria included age under 2 years or over 18 years, hypothyroidism, renal disease, malignancy, treatment with immunosuppressive drugs, connective tissue disease, any cardiovascular event within 6 months, and any acute illness.

The children were divided in 4 groups according to cholesterol serum levels and family history, including hypercholesterolemic children with family history of vascular disease (n = 20), normocholesterolemic children with family history of vascular disease (n = 19), and hypercholesterolemic (n = 21) and normocholesterolemic children (n = 21) without family history of vascular disease. Family history was considered positive only if each study subject’s father or mother had experienced myocardial infarction or stroke at <55 years of age.

To compare the behavior of oxidative stress and sCD40L in children and adults, 80 adult patients with hypercholesterolemia (n = 40) and normocholesterolemia (n = 40) matched for gender and age were included in the study. Both patients and control subjects were recruited from the same geographic area and followed a typical Mediterranean diet. None of the patients had clinical evidence of cardiovascular disease (as shown by clinical history, physical examination, or electrocardiogram), diabetes mellitus, or hypertension. Patients with hypercholesterolemia had not taken any lipid-lowering agents or antiplatelet drugs in the previous 30 days.

Blood samples were obtained from an antecubital vein after an overnight fast. Serum TC, high-density lipoprotein cholesterol, and triglycerides were measured by an Olympus AN 560 apparatus (Olympus Optical Co., Sizuoka, Japan) using an enzymatic colorimetric method; LDL-C levels were calculated according to the Friedwald formula.

**Analysis of sCD40L and 8-OHdG.** Blood samples mixed with 0.13 M Na citrate (ratio 9:1) were taken between 8 and 9 AM from patients who had fasted at least 12 h; samples were immediately centrifuged at 2,000 rpm for 20 min at 4°C, and the supernatant was collected and stored at −80°C until measurement. Plasma levels of sCD40L were measured with a commercial immunoassay (Quantikine CD40 ligand, R&D Systems Inc., Minneapolis, Minnesota). Intra-assay and interassay coefficients of variation were 7% and 9%, respectively.

Serum levels of 8-OHdG were analyzed using a competitive enzyme-linked immunosorbent assay (Bioxynetech 8-OHdG-EIA, OXIS Health Products, Portland, Oregon). Intra- and interassay coefficients of variation were 2.1% and 4.5%, respectively.

**Platelet p38 MAP kinase.** Platelets (2 × 10^8/ml in tyrode buffer) were prepared as previously described (10), stimulated with collagen (6 mg/μl) (3 min at 37°C), and washed and resuspended in a 2X Lysis buffer (5 mM EDTA, 0.15 mol NaCl, 0.1 mol Tris pH 8.0, 1% triton and protease inhibitor cocktail).

Equal amounts of protein (30 μg/lane) estimated by Bradford assay were solubilized in a 2× Laemmli sample buffer containing 2-mercaptoethanol and loaded in a denaturing SDS/10% polyacrylamide gel. Western blot analysis was performed with monoclonal anti-p38 MAP kinase (1 μg/ml) incubated overnight at 4°C. Immune complexes were detected by enhanced chemiluminescence. The rate of
p38 MAP kinase was analyzed by autoradiography. The developed spots were calculated by densitometric analysis on a NIH-Image 1.62f analyzer, and the amount of phosphorylation was determined by dividing the areas of the phosphorylated spots of stimulated platelets by the area of control unstimulated platelets; the value was expressed as arbitrary units (14). Analysis of platelet p38 MAP kinase phosphorylation was performed in a subgroup of children who were willing to provide a further aliquot of blood. The subgroup included children with normal (n = 8, 4 males, 4 females, mean age 8.8 ± 2.1 years) or high (n = 8, 4 males, 4 females, age 9.0 ± 1.7 years) levels of serum cholesterol.

**Statistical analysis.** Comparisons between groups were carried out by Student t test; in case of non-homogeneous variances, as verified by Levene’s test, the nonparametric test (Kolmogorov-Smirnov [z] test) was used. Data are presented as mean ± SE. Proportions and categorical variables were tested by the chi-square test. The correlation analysis was carried out by Pearson’s test. Multiple linear regression analysis was performed using a stepwise selection method and was performed to determine the independent parameters of CD40L and 8-OHdG. Statistical significance was defined as p < 0.05. Statistical analysis was performed with SPSS 13.0 software for Windows (SPSS Inc., Chicago, Illinois).

**Results**

Clinical characteristics of hypercholesterolemic and normocholesterolemic children and adults are reported in Table 1. Serum levels of cholesterol were 156.6 ± 3.0 mg/dl in children with normocholesterolemia and 228.9 ± 5.6 mg/dl in those with hypercholesterolemia (p < 0.001). Serum levels of cholesterol were 162.0 ± 3.8 mg/dl in adults with normocholesterolemia and 270.8 ± 3.5 mg/dl in adults with hypercholesterolemia (p < 0.001).

**Oxidative stress in children with hypercholesterolemia.** Compared to children with normocholesterolemia, those with hypercholesterolemia had higher oxidative stress, as documented by significantly more elevated values of 8-OHdG serum levels (Fig. 1).

This finding was not influenced by the family history for cardiovascular disease in either hypercholesterolemic or normocholesterolemic children (not shown). Oxidative stress was lower in children compared to adults. Thus, children with normal or high levels of serum cholesterol had lower values of 8-OHdG compared with corresponding adults (Fig. 1). Oxidative stress was significantly associated with serum LDL cholesterol in both children (Fig. 2A) and adults with hypercholesterolemia (Fig. 2B). Also, in overall correlation, LDL cholesterol was significantly associated with 8-OHdG (r = 0.506, p < 0.001).

To establish the independent predictors of 8-OHdG among children, we performed a multiple linear regression analysis including the variables linearly associated to the dependent variable (Table 2); the independent predictor variable, in both groups, was only sCD40L (hypercholesterolemic children: B: 1.071; standard error [SE]: 0.188; standardized coefficient beta: 0.679; R² = 0.679; 8-OHdG = 8-hydroxy-2’-deoxyguanosine).

![Figure 1: 8-OHdG Serum Levels in Normocholesterolemic or Hypercholesterolemic Children and Adults](image)

*p < 0.001. 8-OHdG = 8-hydroxy-2’-deoxyguanosine.

**Table 1: Clinical Characteristics of Hypercholesterolemic and Normocholesterolemic Children and Adults**

<table>
<thead>
<tr>
<th></th>
<th>Hypercholesterolemic Children</th>
<th>Normocholesterolemic Children</th>
<th>p Value*</th>
<th>Hypercholesterolemic Adults</th>
<th>Normocholesterolemic Adults</th>
<th>p Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>9.28 ± 0.5</td>
<td>9.02 ± 0.69</td>
<td>NS</td>
<td>54.0 ± 4.9</td>
<td>52.5 ± 5.1</td>
<td>NS</td>
</tr>
<tr>
<td>Gender</td>
<td>18 males, 23 females</td>
<td>27 males, 13 females</td>
<td>NS</td>
<td>20 males, 20 females</td>
<td>20 males, 20 females</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>102.4 ± 1.76</td>
<td>103.1 ± 1.59</td>
<td>NS</td>
<td>127.5 ± 1.5</td>
<td>124.0 ± 1.8</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>70.8 ± 1.2</td>
<td>67.3 ± 1.4</td>
<td>NS</td>
<td>75.3 ± 1.4</td>
<td>74.7 ± 1.5</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>228.9 ± 5.6</td>
<td>156.6 ± 3.0</td>
<td>&lt;0.001‡</td>
<td>270.8 ± 3.5</td>
<td>162.0 ± 3.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>153.9 ± 6.4</td>
<td>95.9 ± 2.8</td>
<td>&lt;0.001‡</td>
<td>187.5 ± 1.8</td>
<td>97.6 ± 2.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>100.8 ± 11.6</td>
<td>77.2 ± 5.5</td>
<td>0.03‡</td>
<td>102.7 ± 3.2</td>
<td>72.5 ± 2.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>sCD40L (ng/ml)</td>
<td>0.55 ± 0.04</td>
<td>0.19 ± 0.03</td>
<td>&lt;0.001</td>
<td>4.28 ± 0.33</td>
<td>2.23 ± 0.15</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>8-OHdG (ng/ml)</td>
<td>0.55 ± 0.06</td>
<td>0.21 ± 0.02</td>
<td>&lt;0.001‡</td>
<td>3.61 ± 0.54</td>
<td>1.23 ± 0.19</td>
<td>&lt;0.001‡</td>
</tr>
</tbody>
</table>

*Hypercholesterolemic versus normocholesterolemic children; †hypercholesterolemic versus normocholesterolemic adults; ‡nonparametric test.

8-OHdG = 8-hydroxy-2’-deoxyguanosine; LDL = low-density lipoprotein; sCD40L = soluble CD40L.
sCD40L and oxidative stress in children with hypercholesterolemia. Compared to children with normocholesterolemia, those with hypercholesterolemia had higher values of sCD40L plasma levels (Fig. 3). This finding was not influenced by family history for cardiovascular disease in either hypercholesterolemic or normocholesterolemic children (data not shown).

Plasma levels of sCD40L were lower in children compared with adults. Thus, children with normal or high levels of serum cholesterol had lower values of sCD40L compared with the corresponding adults (Fig. 3). Soluble CD40L plasma levels significantly correlated with serum LDL cholesterol in both children ($r = 0.766$, $p < 0.001$) (Fig. 4A) and adults with hypercholesterolemia ($r = 0.834$, $p < 0.001$) (Fig. 4B). Also, in overall correlation, LDL cholesterol was significantly associated with sCD40L ($r = 0.539$, $p < 0.001$).

Plasma levels of sCD40L significantly correlated with 8-OHdG in both children ($r = 0.676$, $p < 0.001$) (Fig. 5A) and adults with hypercholesterolemia ($r = 0.832$, $p < 0.001$) (Fig. 5B); also, in overall correlation, LDL cholesterol was significantly associated with sCD40L ($r = 0.856$, $p < 0.001$).

To establish the independent predictors of sCD40L among children, we performed a multiple linear regression analysis including the variables linearly associated to the dependent variable (Table 2). In the group of hypercholesterolemic children, the independent predictor variables were LDL cholesterol ($B: 0.004$; SE: 0.001; standardized coefficient beta: 0.563; $p < 0.001$) and 8-OHdG ($B: 0.676$, $p < 0.001$).
0.235; SE: 0.070; standardized coefficient beta: 0.370; p = 0.002), accounting for 68.3% of the total variability of sCD40L. In normocholesterolemic children sCD40L was independently associated only with 8-OHdG (B: 0.714; SE: 0.063; standardized coefficient beta: 0.878; p < 0.001; R² = 77.1%).

**Phosphorylation of MAP kinase in children with hypercholesterolemia.** Upon stimulation, platelets activate p38 MAP kinase, which is involved in modulating intracellular oxidative stress via activation of NADPH oxidase (14). Thus, we used platelets as a tool to investigate the phosphorylation of p38 MAP kinase in children with normal (150 ± 4.4 mg/dl) or high (233.5 ± 8.7 mg/dl) levels of serum cholesterol. As shown in Figure 6, hypercholesterolemic children showed higher platelet p38 MAP kinase phosphorylation compared with control subjects.

**Discussion**

This study provides evidence that in children with family and polygenic hypercholesterolemia, there is an early increase of oxidative stress that suggests the stress’s potential interplay with CD40L up-regulation.

Experimental and clinical studies provided strong evidence in favor of the pivotal role of oxidative stress in the atherosclerotic process. Several markers of oxidative stress, including circulating antibodies against oxidized LDL, urinary excretion of isoprostanes, and serum and urinary levels of 8-OHdG have been shown to be associated with subclinical and preclinical atherosclerosis and acute coronary syndrome in adulthood (10,16,17).

Children with hypercholesterolemia are at risk of early preclinical atherosclerosis, as shown by the fact that serum cholesterol is a good predictor of intima-media thickness in young adults (4). Cholesterol could promote early in situ oxidative stress with ensuing monocyte recruitment in the sub-endothelium layer. In accordance with this hypothesis, oxidized LDL has been detected in the aortas of human fetuses whose mothers were hypercholesterolemic, even before monocyte infiltration, suggesting that cholesterol may also promote oxidative stress in children with hypercholesterolemia (1). Clinical evidence of early oxidative stress has been documented by an interventional study with vitamins E and C showing that these antioxidant vitamins were able to ameliorate endothelial dysfunction (18). However, this treatment did not change biologic markers of oxidative stress; therefore, it was unclear whether children with hypercholesterolemia had enhanced oxidative stress.
and the stress’s interplay with endothelial dysfunction. More recently Rodenburg et al. (11) found higher levels of apolipoprotein B-immuno complexes and malondialdehyde-LDL autoantibodies in children with familial hypercholesterolemia compared with control subjects, suggesting the existence of an immunologic response to oxidized LDL. Consistent with these findings are the results of the present study showing an enhanced oxidative stress in children with hypercholesterolemia; the significant association between serum levels of 8-OHdG and serum cholesterol is in accordance with a previous study in adults (10) and strongly suggests that cholesterol may also promote oxidative stress in children with hypercholesterolemia.

The increased levels of sCD40L are another finding of the present study that may explain the early atherosclerosis occurring in hypercholesterolemic children. Thus, CD40L is a protein that exerts inflammatory and prothrombotic effects and is implicated in the process of atherosclerosis (12).

Among the several cells expressing CD40L, platelets play an important role as, upon agonist stimulation, they release CD40L into the circulatory system as a soluble form; more than 95% of circulating sCD40L is attributed to its release from activated platelets (19). Previous studies showed enhanced sCD40L and platelet CD40L expression in adults with hypercholesterolemia with a strong correlation between sCD40L and serum cholesterol (20,21), indicating that cholesterol could up-regulate platelet CD40L. Consistent with these data are the results of the present study, showing a significant increase of plasma sCD40L in children with hypercholesterolemia with a significant correlation between this variable and serum cholesterol. This indicates that hypercholesterolemia is associated with early platelet activation, which may contribute to the atherosclerotic process via CD40L up-regulation. To our knowledge, there is only 1 study that measured sCD40L in children with familial hypercholesterolemia and showed normal serum levels compared with control subjects (22). Different study population and, overall, blood sampling may account for these apparent divergent results. In particular, Ueland et al. (22) measured sCD40L in serum, which reflects the global capacity of platelets to produce sCD40L, whereas we measured sCD40L in plasma, which expresses the actual circulating levels of sCD40L. It is, therefore, possible that platelets from children with hypercholesterolemia have a normal intraplatelet content of CD40L but, as a consequence of enhanced activation, they release higher amounts of sCD40L in the circulation.

Previous studies have shown that oxidative stress is implicated in up-regulation of CD40L (20,24,25); in particular, we have documented that in patients with hereditary deficiency of gp91phox, the central core of NADPH oxidase (20), platelet CD40L, was down-regulated, suggesting that NADPH oxidase-generating superoxide anion is crucial for platelet CD40L expression. Therefore, we speculated that children with hypercholesterolemia have an enhanced platelet oxidative stress that could predispose to CD40L up-regulation and ultimately to increased sCD40L. Experiments performed by measuring p38 MAP kinase, a protein that is known to activate NADPH oxidase (14), are in favor of this hypothesis, as a higher phosphorylation of this protein was observed in platelets from hypercholesterolemic children compared to control subjects. Further support to the interplay between oxidative stress and platelet CD40L up-regulation was provided by the significant correlation between serum levels of 8-OHdG and plasma levels of sCD40L. Even we cannot exclude that the significant association between these two variables may be also interpreted as a consequence of the pro-oxidant property of CD40L (10,14); it is likely that in vivo oxidative stress behaves as a stimulus for CD40L regulation. Thus, we have
previously demonstrated that in humans, intravenous infusion of ascorbic acid, a known antioxidant, was associated with platelet CD40L down-regulation (26).

Together, these data indicate that hypercholesterolemia may be responsible for a premature increase of oxidative stress and in turn CD40L up-regulation, thereby contributing to early atherosclerosis. Owing to the fact that our population included both family and polygenic hypercholesterolemia, it is likely that genetic/diet causes play a role in enhancing oxidative stress and sCD40L, but the contribution of each factor should be further analyzed by ad hoc study.

In the present study, we also tested the hypothesis that the familial history of cardiovascular disease could influence oxidative stress and in turn CD40L up-regulation. Recent studies have shown that individuals with family history for cardiovascular disease have early signs of subclinical atherosclerosis such as increased intima-media thickness (13). We speculated that these changes could reflect systemic signs of early oxidative stress and CD40L up-regulation. Even though our findings did not show changes compatible with a role of family history in affecting oxidative stress and sCD40L, the sample size is likely to be too small to test such a hypothesis. Further study is necessary to investigate whether oxidative stress is an independent predictor of family history of atherosclerotic disease.

Our study has potential clinical implications as it suggests the need of early intervention to reduce oxidative stress and its sequelae. Even if short-term treatment with antioxidants improved endothelial dysfunction (18), long-term supplementation with these vitamins proved to be ineffective to prevent atherosclerotic progression and should not be indicated in children with hypercholesterolemia (27). Conversely, appropriate dietary intervention aimed at enhancing the natural defenses against oxidative stress should be explored. For instance, long-term dietary intervention with low-saturated fatty acids has been shown to ameliorate endothelial dysfunction, an effect that was explained in part by cholesterol lowering, but its role on oxidative stress was not investigated (28). Integration of antioxidant nutrients in low-saturated fatty acid diets should be tested to assess whether these diets influence oxidative stress in children with hypercholesterolemia.

This study has potential limitations that should be acknowledged. First, we used only 1 test to measure oxidative stress. However analysis of 8-OHdG proved to be a reliable assay in clinical settings characterized by enhanced oxidative stress such as renovascular disease and hypercholesterolemia (10,16). Also, in another clinical setting, simultaneous measurement of serum levels of 8-OHdG and urinary isoprostanes showed that these two markers of oxidative stress have a parallel behavior (29). Second, analysis of platelet p38 MAP kinase has been performed only in a subgroup of children and, although the increase of its phosphorylation was much higher in hypercholesterolemic children compared with control subjects, we cannot be certain that such differences would persist in analysis of the entire population. Third, the differences between children and adults are consistent with previous studies indicating that oxidative stress and platelet activation increase with advancing age (30–33); however, it is unclear whether this reflects atherosclerotic burden or if age per se is associated with changes of mechanisms controlling for oxidative stress and platelet activation.

In conclusion, we showed an early interplay between oxidative stress and sCD40L in children with hypercholesterolemia, providing a novel insight into a mechanism potentially accounting for premature atherosclerosis in this clinical setting.

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