Sphingolipids Contribute to Human Atherosclerotic Plaque Inflammation

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Objective—Lipids are central to the development of atherosclerotic plaques. Specifically, which lipids are culprits remains controversial, and promising targets have failed in clinical studies. Sphingolipids are bioactive lipids present in atherosclerotic plaques, and they have been suggested to have both proatherogenic and antiatherogenic. However, the biological effects of these lipids remain unknown in the human atherosclerotic plaque. The aim of this study was to assess plaque levels of sphingolipids and investigate their potential association with and contribution to plaque vulnerability.

Approach and Results—Glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and sphingosine-1-phosphate were analyzed in homogenates from 200 human carotid plaques using mass spectrometry. Inflammatory activity was determined by analyzing plaque levels of cytokines and plaque histology. Caspase-3 was analyzed by ELISA technique. Expression of regulatory enzymes was analyzed with RNA sequencing. Human coronary artery smooth muscle cells were used to analyze the potential role of the 6 sphingolipids as inducers of plaque inflammation and cellular apoptosis in vitro. All sphingolipids were increased in plaques associated with symptoms and correlated with inflammatory cytokines. All sphingolipids, except sphingosine-1-phosphate, also correlated with histological markers of plaque instability. Lactosylceramide, ceramide, sphingomyelin, and sphingosine-1-phosphate correlated with caspase-3 activity. In vitro experiments revealed that glucosylceramide, lactosylceramide, and ceramide induced cellular apoptosis. All analyzed sphingolipids induced an inflammatory response in human coronary artery smooth muscle cells.

Conclusions—This study shows for the first time that sphingolipids and particularly glucosylceramide are associated with and are possible inducers of plaque inflammation and instability, pointing to sphingolipid metabolic pathways as possible novel therapeutic targets. (Arterioscler Thromb Vasc Biol. 2016;36:1132-1140. DOI: 10.1161/ATVBAHA.116.305675.)

Key Words: atherosclerosis ■ carotid stenosis ■ inflammation ■ sphingolipids ■ stroke

Oxidized low-density lipoproteins (ox-LDLs) generated through oxidative modification of LDL trapped in the vessel wall are an important contributor to the atherosclerotic process. Besides LDL, several other groups of lipids, such as sphingolipids, have been shown to have potent biological effects in the atherosclerotic process. Ceramides are a group of sphingolipids generated from hydrolysis of sphingomyelin by sphingomyelinases, neutral sphingomyelinases, and acidic sphingomyelinases (A-SMase). Ox-LDL increases the production of ceramides through activation of sphingomyelinases.1,2 The sphingomyelinase-dependent hydrolysis of LDL-sphingomyelin has been proposed as a potential pathway for ceramide accumulation in atherosclerotic plaques.3 Glycosphingolipids, such as glucosylceramide and lactosylceramide, generated from ceramides (Figure 1), have also been found in high concentrations in human atherosclerotic plaques compared with healthy vascular tissue.3,4 Both lactosylceramide and ceramide have been shown to be involved in cell apoptosis through the action of neutral sphingomyelinase,5,6 but lactosylceramide has also been suggested to contribute to plaque formation by stimulating cell proliferation in human aortic smooth muscle cells.6

Glucosylceramide, lactosylceramide, and ceramide have also been suggested to have proinflammatory properties. Accumulation of ceramides induces a systemic inflammatory response, and lactosylceramide upregulates

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adhesion molecules on vascular endothelial cells and activates neutrophils, thereby possibly contributing to plaque inflammation.9–13

Furthermore, a study by Bietrix et al14 suggested glucosylceramide synthesis as a possible therapeutic target for inhibiting the atherosclerotic process in mice. By inhibiting glucosylceramide synthase, the inflammatory gene expression was downregulated and the atherosclerotic plaque formation decreased.

Although sphingolipids levels have been shown to be higher in atherosclerotic plaques compared with the normal artery, it remains unknown whether there is a difference in the levels of sphingolipids between plaques associated with symptoms and plaques not associated with symptoms. It also remains unknown whether these lipids contribute to plaque progression and vulnerability.

The aim of this study was to assess plaque content of the different sphingolipids glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and S1P and to investigate their association and role in plaque inflammation and vulnerability.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

### Results

To elucidate whether the amount of specific lipids in human atherosclerotic plaques is associated with a plaque phenotype prone to cause symptoms, 200 carotid plaques from the Carotid Plaque Imaging Project were analyzed. Clinical characteristics of the cohort are summarized in Table 1 and further described in the Materials and Methods section in the online-only Data Supplement. Lipids were analyzed using mass spectrometry. Glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and S1P are presented as a total content of all measured subspecies (different chain lengths are presented in Table I in the online-only Data Supplement).

**Increased Sphingolipids in Symptomatic Patients**

Glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and S1P were all significantly increased in plaques associated with symptoms compared with plaques from asymptomatic patients (264.5 interquartile range [IQR], 183.0–343.6] versus 212.4 [IQR, 162.2–296.6] nmol/g wet weight plaque, \( P<0.05 \); 138.6 [IQR, 87.4–189.3] versus 105.4 [IQR, 70.5–151.1] nmol/g, \( P<0.005 \); 121.7 [IQR, 79.4–165.5] versus 95.8 [IQR, 70.5–124.9] nmol/g, \( P=0.001 \); 5.7 [IQR, 3.5–8.6] versus 3.8 [IQR, 2.3–6.0] nmol/g, \( P<0.001 \); 6098.6 [IQR, 4638.4–8002.6] versus 5310 [IQR, 4093.1–6252.7] nmol/g, \( P<0.005 \); and 0.34 [IQR, 0.25–0.42] versus 0.29 [IQR, 0.21–0.36] nmol/g, \( P=0.01 \), respectively; Figure 2A–2F). These differences in sphingolipids in symptomatic compared with asymptomatic plaques remained significant even after adjusting for the use of statins and the presence of diabetes mellitus type 2.

**Sphingolipid and Plaque Composition**

Glucosylceramide, lactosylceramide, and dihydroceramide correlated with plaque content of macrophages (CD68), as well as with lipids (oil red O; Table 2). Sphingomyelin and ceramide were also found to be positively correlated with plaque levels of lipids (Table 2). Plaque levels of ox-LDL correlated only with

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**Figure 1.** Studied pathways of sphingolipid metabolism. Cer indicates ceramide; dhCer, dihydroceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; S1P, sphingosine-1-phosphate; and SM, sphingomyelin.
ceramide and dihydroceramide (r=0.148, P=0.038; r=0.168, P=0.018, respectively). Lactosylceramide did not show a significant correlation to ox-LDL although a tendency was found (r=0.134; P=0.06). Lactosylceramide, ceramide, dihydroceramide, and sphingomyelin correlated inversely with plaque levels of vascular smooth muscle cells (VSMCs; r=−0.195, P=0.006; r=−0.175, P=0.014; r=−0.232, P=0.001; and r=−0.154, P=0.031, respectively). S1P did not correlate with histological markers of plaque vulnerability or stability.

Sphingolipids and Inflammation
As markers of plaque inflammation, besides assessing macrophages, inflammatory cytokines and chemokines were measured. Glucosylceramide, lactosylceramide, dihydroceramide, and sphingomyelin correlated with plaque content of the proinflammatory cytokines monocyte chemotactant protein-1, interleukin-6 (IL-6), and macrophage inflammatory protein-1β (MIP-1β; Table 3). Ceramide correlated with IL-6 and MCP-1 but not MIP-1β. S1P correlated with tumor necrosis factor-α (TNF-α) and regulated on activation, normal T cell expressed and secreted (RANTES). When grouping plaques into symptomatic and asymptomatic, the same patterns of associations were found although the majority did not reach level of significance (Table IIA and IIB in the online-only Data Supplement). None of the analyzed sphingolipids were expressed and secreted (RANTES). When grouping plaques and the effect on apoptosis was analyzed after 48 hours (Figure 4). At 0.1 µmol/L, a low concentration compared with median concentrations present in the plaque (Table 4), cell viability was not affected. At concentrations of 1 and 10 µmol/L glucosylceramide, lactosylceramide, and ceramide were found to induce apoptosis in HCASMCs (Figure 4A), whereas no effects were seen for dihydroceramide, sphingomyelin, and S1P.

At high concentrations (10 µmol/L), glucosylceramide, lactosylceramide, and ceramide were found to induce cellular necrosis as measured by lactate dehydrogenase (Figure 4B).

Sphingomyelinase Localization and Expression
A-SMase localization was determined histologically (Figure 5A and 5B). The methods available for SMase analysis in plaque tissue are limited, and further functional analysis was not performed. However, according to histological comparisons, a colocalization was shown between A-SMase,

### Table 1. Clinical Characteristics of Patients (n=197)

<table>
<thead>
<tr>
<th></th>
<th>AS</th>
<th>S</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>66.9 (SD, 6.7)</td>
<td>71.7 (SD, 8.8)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>80 (n=76)</td>
<td>73 (n=77)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>23 (n=22)</td>
<td>42 (n=44)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Smoking (past or currently), %</td>
<td>84 (n=80)</td>
<td>80 (n=83)</td>
<td>NS</td>
</tr>
<tr>
<td>Statin treatment, %</td>
<td>92 (n=87)</td>
<td>82 (n=86)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.3 (IQR, 3.5–5.1)</td>
<td>4.3 (IQR, 3.6–5.2)</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>2.5 (IQR, 1.8–3.1)</td>
<td>2.5 (IQR, 2–3.3)</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.1 (IQR, 0.85–1.4)</td>
<td>1.1 (IQR, 0.9–1.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides cholesterol</td>
<td>1.3 (IQR, 0.93–1.8)</td>
<td>1.2 (IQR, 0.9–1.7)</td>
<td>NS</td>
</tr>
<tr>
<td>White blood cell count, 10^9/L</td>
<td>7.9 (SD, 1.9)</td>
<td>7.9 (SD, 2.1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

AS indicates asymptomatic patients; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; NS, nonsignificant; and S, symptomatic patients. P value represents the significance comparing the symptomatic and asymptomatic group of patients. Normally distributed variables are presented as mean±SD, and non-normally distributed variables are presented as median and IQR.

Sphingolipids Induce Apoptosis and Cell Death in HCASMCs
In plaque tissue, the effector caspase, caspase-3, activity was analyzed as a marker of cellular apoptosis. Lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and S1P were all positively associated with plaque levels of caspase-3 (r=0.225, P=0.001; r=0.213, P=0.002; r=0.32, P<0.001; r=0.164, P=0.021; and r=0.199, P=0.005, respectively). An association between caspase-3 levels and glucosylceramide (r=0.133; P=0.06) was also found, but it did not reach statistical significance.

As inverse associations were found between sphingolipids and VSMCs, as well as positive associations between sphingolipids and caspase-3 in plaque tissue, we further aimed to investigate how sphingolipids affect cell viability of stabilizing cells within the plaque. HCASMCs were subjected to increasing concentrations of sphingolipids, and the effect on apoptosis was analyzed after 48 hours (Figure 4). At 0.1 µmol/L, a low concentration compared with median concentrations present in the plaque (Table 4), cell viability was not affected. At concentrations of 1 and 10 µmol/L glucosylceramide, lactosylceramide, and ceramide were found to induce apoptosis in HCASMCs (Figure 4A), whereas no effects were seen for dihydroceramide, sphingomyelin, and S1P.

Activation of Inflammation In Vitro
The association found between sphingolipids and inflammatory cytokines suggest a proinflammatory role of sphingolipids within the plaque. To investigate how sphingolipids affect cytokine release, human coronary artery smooth muscle cells (HCASMCs) were incubated with glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and S1P at 4 different concentrations (0.01, 0.1, 1, and 10 µmol/L) for 24 hours, and the effect on IL-6 release into the media was assessed. All sphingolipids were found to induce a significant release of IL-6 from HCASMCs compared with control cells (Figure 3A). For S1P, we could see an effect on IL-6 release only at concentrations above 1 µmol/L, which may not be clinical relevant considering the generally low concentrations of S1P in plaque tissue (Table 4). In an additional experiment, we investigated the effect of lactosylceramide and glucosylceramide on the release of the other cytokines found to be significantly associated with the sphingolipids in the plaque homogenates. In this experiment, glucosylceramide was found to induce the release of MCP-1, MIP-1β, RANTES, and TNF-α from HCASMCs (Figure 3B–3E). None of the sphingolipids showed an effect in vitro on macrophage IL-6 release (data not shown).
macrophages, and lipids, strengthening the hypothesis that sphingolipid metabolism is a contributing factor in atherosclerotic inflammation.

Gene Expression of Enzymes in Sphingolipid Pathways
No difference in gene expression was observed in plaque RNAseq data for enzymes involved in sphingolipid and glycosphingolipid pathways when plaques from asymptomatic patients were compared with plaques from patients with a recent cerebrovascular event (n=8 versus n=8; Table III in the online-only Data Supplement).

Sphingolipids and Clinical Characteristics
Ceramide levels in plaques were significantly increased in plaques from diabetics compared with nondiabetics (121 [IQR 88–162.9] versus 98.5 [IQR 68.7–144.8]; P=0.029). Glucosylceramide, sphingomyelin, and S1P correlated with plasma levels of LDL (r=0.196, P<0.01; r=0.222, P<0.005; and r=0.155, P<0.05, respectively). Ceramide, dihydroceramide, and lactosylceramide also correlated with age (r=0.304, P<0.001; r=0.26, P<0.001; and r=0.196, P<0.01, respectively). No differences in sphingolipid levels were found when comparing patients with or without statin treatment.

Table 2. Associations Between Histological Features of Plaque Vulnerability and Plaque Levels of GlcCer, LacCer, Cer, dhCer, SM, and S1P

<table>
<thead>
<tr>
<th>Feature</th>
<th>GlcCer</th>
<th>LacCer</th>
<th>Cer</th>
<th>dhCer</th>
<th>SM</th>
<th>S1P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipids (Oil Red O)</td>
<td>r=0.391*</td>
<td>r=0.497*</td>
<td>r=0.321*</td>
<td>r=0.462*</td>
<td>r=0.362*</td>
<td>NS</td>
</tr>
<tr>
<td>Macrophages (CD68)</td>
<td>r=0.203*</td>
<td>r=0.255*</td>
<td>NS</td>
<td>r=0.239*</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Significance adjusted for multiple correlations using Bonferroni correction and marked by *P<0.004. Cer indicates ceramide; dhCer, dihydroceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; NS, nonsignificant; S1P, sphingosine-1-phosphate; and SM, sphingomyelin.
Discussion

Recent data highlighted sphingolipids as potential therapeutic targets in the atherosclerotic process. Sphingolipids are increased in human plaque tissue, and in vitro studies have shown possible effects of these lipids on the atherosclerotic process. However, no studies have studied their association to atherosclerotic plaque phenotype and their potential role as proinflammatory mediators in human atherosclerosis.

Sphingolipids and Plaques Associated With Symptoms

All 6 analyzed sphingolipids were increased in plaque tissue associated with symptoms, suggesting a possible relationship between these sphingolipids and plaque vulnerability. Glucosylceramide and lactosylceramide have in previous studies been shown to be increased in human atherosclerotic plaques, but differences between symptomatic and asymptomatic plaques have not previously been studied. However, the increased levels of sphingolipids observed in the symptomatic plaques do not tell us whether sphingolipids contribute to plaque instability or whether they result from increased inflammatory activity after the event of a plaque rupture.

Sphingolipids and Atherosclerotic Plaque Inflammation

To assess plaque inflammation, the content of macrophages in plaques was determined histologically, and plaque content of cytokines was measured in plaque homogenates. Glucosylceramide, lactosylceramide, and dihydroceramide correlated with plaque content of macrophages. The positive association between glucosylceramide and macrophages may be explained by the fact that glucosylceramide levels are high in human plaque monocytes as shown by Chatterjee et al. Accordingly, it has been shown that ox-LDL increases macrophage lactosylceramide synthesis, but the association with lactosylceramide and macrophages may also be the effect of enhanced macrophage migration because of lactosylceramide-dependent induction of adhesion molecule expression on monocytes and endothelial cells. Because histological measurement of macrophages on cryosections from the 1-mm thick fragments of the most stenotic region of a plaque is a blunt, limited way to study inflammation, we also evaluated cytokine levels in the majority of the plaque tissue. This provided a more complete characterization of the inflammatory activity within the plaque and showed that all analyzed sphingolipids, with the exception of S1P, correlated with several proinflammatory cytokines and in general most strongly to MCP-1, MIP-1β, and IL-6. Yet, S1P correlated positively with the proinflammatory cytokines RANTES and TNF-α.

Sphingolipids, Neutral Lipids, and Ox-LDL

To further evaluate the association of sphingolipids and plaque instability, the content of neutral lipids (oil red O) and ox-LDL were measured histologically. The vulnerable plaque is known to be rich in ox-LDL. Ox-LDL increases matrix metalloproteinase-2 activity, an enzyme that is highly present in plaques and activates neutral sphingomyelinase in human aortic smooth muscle cells, which in turn activates the sphingomyelin/ceramide pathway resulting in enhanced sphingomyelin hydrolysis into ceramide and eventually S1P. However, in this study, ceramide correlated with plaque content of ox-LDL, LDL, and sphingomyelin (r=0.836; P<0.001). Additional analyses to evaluate plaque A-SMase were performed, but beyond the location of the enzyme, we were not able to distinguish differences in enzyme activity.

Studies by Chatterjee et al. have also shown that the biosynthesis of lactosylceramide from glucosylceramide is regulated by LDL and ox-LDL levels. Higher levels of LDL suppress lactosylceramide synthase, an enzyme promoting synthesis of more complex glycosphingolipids from glucosylceramide (Figure 1), which thereby inhibits synthesis of lactosylceramide from glucosylceramide.

Both lactosylceramide and glucosylceramide correlated with plaque content of lipids in the present study. Despite not reaching significance (P=0.06), a tendency was found between plaque content of ox-LDL and lactosylceramide, fitting with the idea of ox-LDL promoting lactosylceramide formation.

Sphingolipids as Possible Inducers of Inflammation In Vitro

To investigate whether the measured sphingolipids induced an inflammatory response in vitro, HCASMCs were incubated with the different sphingolipids in increasing concentrations. All 6 sphingolipids induced a significant release of IL-6 from HCASMCs in high concentration (10 μmol/L), consistent with the correlations found in plaque homogenates. Glucosylceramide, lactosylceramide, dihydroceramide, sphingomyelin, and S1P were also shown to induce IL-6 release.
already at concentrations of 1 \( \mu \text{mol/L} \). Additional experiment also confirmed that glucosylceramide induced a significant release of MIP-1\(\beta\), TNF-\(\alpha\), MCP-1, and RANTES from HCASMCs. Interestingly, glucosylceramide induced the release of these cytokines even at relatively low concentrations when compared with the median plaque concentrations of glucosylceramide.

Few studies have investigated the associations between ceramide, glycosphingolipids, and atherosclerotic plaque inflammation. However, some evidence supports the associations found in this study. Accordingly, the levels of MCP-1, a strong inducer of monocyte migration, has been shown to be increased in mouse models accumulating ceramide.9 Furthermore, Bietrix et al14 showed that inhibition of glucosylceramide synthase decreased TNF-\(\alpha\) and MCP-1 mRNA expression. The hypothesis of glucosylceramide as an inducer of TNF-\(\alpha\) and MCP-1 expression/release is supported by our findings. Glucosylceramide correlated with both TNF-\(\alpha\) and MCP-1 in plaque homogenates and induced a release of these cytokines in vitro.

Although sphingolipids were only shown to induce an inflammatory response in HCASMCs and not in human...
macrophages, this does not rule out their potential contribution to atherosclerotic plaque inflammation. According to the present ex vivo and in vitro findings, sphingolipids do seem to play an important role in atherosclerotic inflammation. As the VSMCs are located in the media or in the cap near the endothelium and the arterial lumen, the release of cytokines from these cells would induce an inflammatory activated state in the endothelium and stimulate inflammatory cell migration. This suggests that sphingolipids may contribute to an early inflammatory response and attract inflammatory cells to the site of atherosclerotic plaque development rather than activating monocytes and macrophages within the atherosclerotic tissue.

**Apoptosis of VSMCs Is Induced by Sphingolipids**

VSMCs are important for maintaining plaque stability. The loss of stabilizing VSMC is one of the important features of the vulnerable plaque leading to a thin protective cap and risk for developing a subsequent plaque rupture.

Whether ceramide and lactosylceramide can be considered as inducers of apoptosis is controversial. As mentioned, lactosylceramide has been shown to induce proliferation in human aortic smooth muscle cells; conversely, there is also evidence for lactosylceramide and ceramide as mediators of SMase-induced apoptosis in human osteosarcoma cell lines and endothelial cells. According to our results, ceramide and lactosylceramide were negatively associated with plaque content of VSMCs. These findings were further supported by the positive correlation found between ceramide, lactosylceramide, and plaque levels of caspase-3. Although a tendency toward increased plaque levels of glucosylceramide and caspase-3 levels was found, the association did not reach statistical significance.

To evaluate the effect on apoptosis, HCASMCs were incubated with the different sphingolipids in increasing concentrations. At concentrations of 1 and 10 \( \mu \text{mol/L} \), glucosylceramide, lactosylceramide, and ceramide were shown to induce apoptosis, whereas only concentrations of 10 \( \mu \text{mol/L} \) induce cellular necrosis.

The levels of sphingolipids needed for inducing apoptosis in vitro are generally higher than the median levels of sphingolipids in the plaque tissue. However, it is difficult to compare concentrations in plaque tissue and in cell culture medium.

**Figure 4.** A, Glucosylceramide (GlcCer), lactosylceramide (LacCer), and ceramide (Cer) induced apoptosis in human coronary arterial smooth muscle cells (HCASMCs) in a concentration-dependent manner. B, High levels (10 \( \mu \text{mol/L} \)) of GlcCer, LacCer, and Cer also caused necrosis in HCASMCs. *\( P<0.05 \); **\( P<0.01 \); ***\( P<0.005 \). dhCer indicates dihydroceramide; S1P, sphingosine-1-phosphate; and SM, sphingomyelin.

**Figure 5.** A, Histological staining for acidic sphingomyelinase (A-SMase), macrophage (CD68), and lipid (oil red O [ORO]) location and the presence in the human atherosclerotic plaque. B, Amplifications of the inset showing colocalization between SMase, CD68, and ORO, as well the negative control for A-SMase. Scale bars, 2 mm (A) and 300 \( \mu \text{m} \) (B).
Considering the median levels of glucosylceramide, lactosylceramide, and ceramide (0.25, 0.12, and 0.11 µmol/g wet weight plaque, respectively) and the concentrations needed to induce cellular apoptosis and IL-6 release (1–10 µmol/L), it cannot be excluded that the used concentrations are of biological relevance. Furthermore, knowing that the atherosclerotic plaque is a heterogeneous tissue, it is likely that the levels of sphingolipids will differ in different locations within the tissue, and local concentrations of sphingolipids may be several times higher than the median homogenate levels at some locations.

**S1P Does Not Associate With Plaque Inflammation or Vulnerability**

In contrast to the other investigated sphingolipids, S1P has been suggested to have antiatherogenic as opposed proatherogenic properties. In plaque tissues, S1P did not correlate with either histological markers of plaque vulnerability or inflammation. When analyzing plaque cytokine levels, S1P was significantly correlated with TNF-α and RANTES. However, S1P failed to induce an inflammatory cytokine release from HCASMCs in vitro. Only at a high concentration (1 and 10 µmol/L), in comparison to median plaque levels (3.1x10^{-4} µmol/g), was an increase in IL-6 release detected, suggesting that the effect on IL-6 release is not biologically relevant in vivo.

Interestingly, S1P was the only sphingolipid that was positively associated with the growth factor PDGF in the plaque tissue. PDGF stimulated VSMC growth and collagen production and is generally considered to contribute to a stable plaque phenotype. PDGF may also stimulate S1P production by activating sphingosine kinase leading to increased S1P levels. S1P was unexpectedly found to correlate with caspase-3 levels. On the contrary, S1P did not induce HCASMC apoptosis in the in vitro experiments, not even at high concentrations. It has been suggested that an imbalance in ceramide/S1P levels could differentiate between cellular apoptosis and survival. Considering the median ceramide/S1P ratio (109/0.31 nmol/g), this could be a possible explanation. However, it could be argued that this theory should show an inverse association rather than a positive association between S1P and caspase-3. Furthermore, ceramide and S1P levels correlated positively (r=0.321; P<0.001), which may not support this theory although it does not exclude it.

Taken together, the present findings suggest that S1P levels may be associated with cellular apoptosis but does not contribute to it.

**mRNA Expression of Sphingolipid Pathway Enzymes**

As the synthesis and degradation of sphingolipids are tightly regulated by a broad spectrum of enzymes, RNA sequencing was performed to investigate whether an increase in plaque levels of sphingolipids and glycosphingolipids could be explained by differential expression of genes coding for enzymes involved in relevant pathways. In the current analysis, no significant differences in the expression of these enzymes were observed. This does not rule out the role of these enzymes in plaque progression, as enzyme activity or localization could also be involved and remain to be evaluated.

**Study Limitations**

The histological analysis of A-SMase performed in the present study is limited by the fact that the method does not allow SMase activity assessment. However, the additional in vitro experiments provide evidence that the analyzed lipids are important for plaque inflammation and the induction of cellular apoptosis.

Furthermore, no differential expression of enzymes was observed, which may be a power issue because of the small number of plaques used for RNA sequencing.

**Conclusions**

In conclusion, this is the first study to demonstrate that glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and S1P are increased in human atherosclerotic plaques associated with symptoms. The current analysis also provides evidence that ceramide, lactosylceramide, and particularly glucosylceramide are not only associated with plaque inflammation and vascular smooth muscle apoptosis but also contribute to it. Our findings suggest an important role for sphingolipids in vulnerable plaque formation and that inhibiting specific targets in sphingolipid formation may, therefore, be a potential therapeutic target. In addition, sphingolipids should be further evaluated as possible biomarkers of plaque vulnerability.

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**Disclosures**

None.

**References**


Lactosylceramide, ceramide, sphingomyelin, and sphingosine-1-phosphate correlated with caspase-3 activity, and glucosylceramide, lactosylceramide, and ceramide induced cellular apoptosis in human coronary arterial cells in vitro.

Sphingolipids correlated with inflammatory cytokines in the human plaque tissue and histological markers of plaque vulnerability.

Glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and sphingosine-1-phosphate were increased in human atherosclerotic plaques associated with symptoms.

Sphingolipids correlated with inflammatory cytokines in the human plaque tissue and histological markers of plaque vulnerability.

Glucosylceramide, lactosylceramide, ceramide, dihydroceramide, sphingomyelin, and sphingosine-1-phosphate induced an inflammatory response in human coronary arterial smooth muscle cells.

Lactosylceramide, ceramide, sphingomyelin, and sphingosine-1-phosphate correlated with caspase-3 activity, and glucosylceramide, lactosylceramide, and ceramide induced cellular apoptosis in human coronary arterial cells in vitro.
Sphingolipids Contribute to Human Atherosclerotic Plaque Inflammation
Andreas Edsfeldt, Pontus Dunér, Marcus Stählinan, Ines G. Mollet, Giuseppe Asciutto, Helena Grufman, Mihaela Nitulescu, Ana Flor Persson, Rachel M. Fisher, Olle Melander, Marju Orho-Melander, Jan Borén, Jan Nilsson and Isabel Gonçalves

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The sphingolipids glucosylceramide (GlcCer), lactosylceramide (LacCer), ceramide (Cer), dihydroceramide (dhCer), sphingomyelin (SM) and sphingosine-1-phosphate (S1P) are all associated to and may contribute to a vulnerable atherosclerotic plaque phenotype, with enhanced inflammatory activity and cellular apoptosis. All six sphingolipids induced IL-6 release from human coronary arterial smooth muscle cells (HCASMC) and GlcCer also induced the release of TNF-α, MIP-1β, MCP-1 as well as RANTES in vitro. Furthermore, LacCer, GlcCer and Cer induced cellular apoptosis in HCASMCs in vitro.
Supplementary Tables
**Supplementary Table I.** Showing ceramides detected on a Quattro Premier (Waters, Milford, USA) triple quadrupole using MRM-transitions.

### MS parameters

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<th>Cone (V)</th>
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**Period 2-Time 4.2-14.0 min**

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**Supplementary Table II a.** Correlations between plaque levels of glucosylceramide (GlcCer), lactosylceramide (LacCer), ceramide (Cer), dihydroceramide (dhCer), sphingomyelin (SM), sphingosine-1-phosphate (S1P) and plaque content of cytokines in asymptomatic plaques.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>GlcCer</th>
<th>LacCer</th>
<th>Cer</th>
<th>dhCer</th>
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<tr>
<td>MCP-1</td>
<td>NS</td>
<td>r=0.443 *</td>
<td>NS</td>
<td>r=0.334 ***</td>
<td>r=0.372 *</td>
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<tr>
<td>MIP-1β</td>
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<td>RANTES</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>r=0.337 *</td>
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<tr>
<td>TNF-α</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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Significance adjusted for multiple correlations according to Bon Ferroni model and marked by * $P<0.002$. NS, Non-significant.
**Supplementary Table II b.** Correlations between plaque levels of glucosylceramide (GlcCer), lactosylceramide (LacCer), ceramide (Cer), dihydroceramide (dhCer), sphingomyelin (SM), sphingosine-1-phosphate (S1P) and plaque content of cytokines in symptomatic plaques.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>GlcCer</th>
<th>LacCer</th>
<th>Cer</th>
<th>dhCer</th>
<th>SM</th>
<th>S1P</th>
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</thead>
<tbody>
<tr>
<td>IL-6</td>
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<td>$r=0.319^*$</td>
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<td>MCP-1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>$r=0.339^*$</td>
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<td>NS</td>
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<tr>
<td>MIP-1β</td>
<td>NS</td>
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<td>NS</td>
<td>$r=0.307^*$</td>
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<td>RANTES</td>
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<td>NS</td>
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<td>NS</td>
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<td>TNF-α</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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Significance adjusted for multiple correlations according to Bon Ferroni model and marked by $^* P<0.002$. NS, Non-significant.
Supplementary Table III

Gene expression of enzymes isoforms involved in the core ceramide/sphingolipid/glycosphingolipid pathway evaluated from global transcriptome RNAseq data collected from 16 plaques: eight asymptomatic donors versus eight donors with a recent stroke even. Statistics of differential expression performed with R package EdgeR.

<table>
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<th>Reaction</th>
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<td>Sphingosine (Cer) → Ceramide (Cer)</td>
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<td>2.3.1.24</td>
<td>Ceramide Synthase 6</td>
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<td>6,0213</td>
<td>0,518</td>
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<tr>
<td>Sphingosine (Cer) → Ceramide (Cer)</td>
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<td>2.3.1.24</td>
<td>Ceramide Synthase 5</td>
<td>0,2018</td>
<td>5,9355</td>
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<td>Ceramide (Cer) → Sphingomyelin</td>
<td>SMPD1</td>
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<tr>
<td>Ceramide (Cer) → Sphingomyelin</td>
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<td>2.3.1.24</td>
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<td>0,1877</td>
<td>5,0970</td>
<td>0,919</td>
<td>1,00</td>
</tr>
</tbody>
</table>

Statistics of differential expression performed with R package EdgeR.
Reference

Methods

Patients

Two hundred human carotid plaques were collected. One hundred and five plaques were associated with symptoms (TIA, stroke or amaurosis fugax) and >70% stenosis, as assessed by ultrasound. Ninety-five plaques were from asymptomatic patients but caused >80% of stenosis. Of the 200 collected plaques, 66 were taken from patients with diabetes (3 patients with type 1 diabetes mellitus and 63 patients with type 2 diabetes). Three patients were operated bilaterally. Cardiovascular risk factors such as hypertension (systolic blood pressure >140mm Hg), smoking (in the past or current), age, diabetes and medications (statin treatment) were recorded. The blood levels of white blood cells, cholesterol, triglycerides, LDL and HDL were registered the day before operation. Clinical characteristics are summarized in Table I. All patients were preoperatively assessed by a neurologist. Informed consent was given by each patient. The study was approved by the local ethical committee.

Sample preparation

Human carotid plaques were collected at endarterectomy and snap-frozen in liquid nitrogen. One-millimetre-thick fragments from the most stenotic region of the frozen plaques were removed for histology. The rest of the plaque was weighed and homogenized in 5 mL of a homogenization buffer consisting of 50 mmol/L Tris-HCl (pH 7.5), 0.25 mol/L sucrose, 2 mmol/L tris(2-carboxyethyl)phosphine HCl, 50 mmol/L NaF, 1 mmol/L Na-orthovanadate, 10 mmol/L Na-glycerophosphate, 5 mmol/L Na-pyrophosphate, protease inhibitor cocktail (Roche Complete, EDTA-free), 1 mmol/L benzamidine, and 10 mmol/L phenylmethylsulfonyl fluoride, as previously described.

Histology

Sections (8 µm) were fixed with Histochoice (Amresco, Solon, OH), dipped in 60% isopropanol, and then in 0.4% Oil Red O in 60% isopropanol (for 20 min) to stain lipids. When staining for macrophages, primary antibody monoclonal mouse anti-human CD68 (DakoCytomation, Glostrup, Denmark), diluted in 10% rabbit serum 1:100, and secondary antibody polyclonal rabbit anti-mouse (DakoCytomation, Glostrup, Denmark), dilution 1:200 in 10% of rabbit serum, were used. When staining for vascular smooth muscle cells (α-actin) primary antibody monoclonal mouse anti-human smooth muscle actin clone 1A4 (DakoCytomation, Glostrup, Denmark), diluted in 10% rabbit serum 1:50, and secondary antibody biotin rabbit anti-mouse Ig (DakoCytomation, Glostrup, Denmark), dilution 1:200 in 10% of rabbit serum.

For aldehyde-modified apoB100 amino acids 661–680 staining, a primary mouse–human chimera of 2D03 anti-ox-LDL antibody with murine constant regions and human variable regions was used, C-2D03. The secondary antibody was F(ab)2 biotinylated polyclonal rabbit anti-mouse (DakoCytomation) at a dilution of 1:500 (1.58 μg/mL) in 10% rabbit-serum in PBS, as previously described.

For SMase analysis Abcam rabbit anti human Anti-Acid sphingomyelinase antibody (Abcam, ab 83354) were used.

Areas of the different stainings in the plaque (% area) were quantified blindly using Biopix iQ 2.1.8 (Gothenburg, Sweden) after scanning with ScanScope Console Version 8.2 (LRI imaging AB, Vista Californien, USA) and photographed with Aperio image scope v.8.0 (Aperio, Vista Californien, USA).

Cytokine assessment

For cytokine and chemokine analysis, aliquots (50 µL) of plaque homogenate were centrifuged at 13000 g for 10 minutes. Twenty-five µL of the supernatant was removed and used for measuring interleukin (IL)-10, IL-6, monocyte chemoattractant protein (MCP-1), macrophage inflammatory protein-1β (MIP-1β), platelet-derived growth factor-AB/BB (PDGF-AB/BB), Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) and tumor necrosis factor-α (TNF-α). The procedure was performed according to the manufacturer’s instructions (Human Cytokine/chemokine immunoassay, Millipore Corporation, MA, USA) and analyzed with Luminex 100 IS 2.3 (Austin, Texas, USA), as previously described.
**Sphingolipid assessment**

Human plaque homogenate (50µl) were automatically extracted using the butanol and methanol method as previously described. Internal standards (SM 17:0, Cer 17:0, GlcCer 12:0 and LacCer 12:0; Avanti lipids, Alabaster, AL, USA) were added during extraction. Sphingolipids Cer, dhCer, GluCer and LacCer were analyzed using high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) with a procedure based on a previously published method. Briefly, a fraction of the total lipid extract was evaporated and reconstituted in isohexane:isopropanol [95:5] and 5µL were injected onto a Sunfire (2.1 x 150 mm, 3µm) straight phase column (Waters, Milford, USA). The HPLC solvent system was composed of isohexane:isopropanol:formic acid [95:5:0.1] as the A-phase and isohexane:isopropanol:50mM ammonium formate [25:65:10] as B-phase. After 1 minute at 100% A-phase the sphingolipids were separated and eluted using a linear gradient to 100%B over 5 minutes. The flow rate was 500µL/min. To increase the detection sensitivity, a post-column infusion of methanol:isopropanol [50:50] with 2.5 mmol/L ammonium formate was made at 100 µL/min. The ceramides were detected on a Quattro Premier (Waters, Milford, USA) triple quadrupole using MRM-transitions according to supplemental table I. Quantification of Cer and dhCer was made using external standards while quantification of GlcCer and LacCer was made against their internal standard. For SM analysis, the total extracts were infused directly into a QTRAP 5500 (ABSciex, Concord Canada) equipped with a robotic nanoflow ion source TriVersa NanoMate (Advion Biosciences, Ithaca, NY, USA) according to previous work.

For S1P analysis, 200µl methanol (containing 100nM 13C2D2-labelled S1P; Toronto Research Chemicals, Toronto, ON, USA) was added to 50µl plaque homogenate. The samples were mixed vigorously for 10 minutes and then centrifuged for 10 min at 20 000 g. The supernatant was evaporated under a stream of nitrogen and then reconstituted in 50µl methanol:acetonitrile:water:formic acid [50:25:25:0.1] and analyzed using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) according to previous work.

**Cell cultures**

Cer, dhCer, GlcCer, LacCer and SM were purchased from Larodan Fine Chemicals AB (Malmö, Sweden) and S1P was purchased from (Sigma Aldrich, Germany). Human monocyte-like cell line THP-1 was cultured in RPMI (GIBCO) supplemented with 10% fetal calf serum, L-Glutamine (2 mM) and were differentiated into macrophages with phorbol myristate acetate (PMA, 50gg/ml) during 72 hours. Human coronary artery smooth muscle cells (HCASMC) were cultured in M231-500 (GIBCO) medium with Smooth Muscle Growth Supplement additives (GIBCO) until the start of experiments. During the experiments THP-1 derived macrophages and HCASMC received medium without additives. HCASMC cells were stimulated with Cer, dhCer, GlcCer, LacCer, SM, S1P or PBS for 48 h. IL-6 was measured with ELISA (BioLegend) according to the manufacturer’s protocol in the media after 24 h. After 48 h stimulation, cells were either stained with Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend) according to manufacturer's protocol or the cells was lysed in cell lysis buffer (BD Pharmingen) and cell protein concentrations were determined with BCA™ protein Assay Kit (Pierce). Cytotoxicity of sphingolipids was measured by lactate dehydrogenase (LDH) release into the medium using Cytotoxicity Detection Kit (Roche). Measurements of Annexin V stained cells were performed using a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA) and analyzed with Summit V4.3 software (Dako). Single stained samples were used to correct for fluorescence spillover in multicolor analyses, and gate boundaries were set by fluorescence-minus-one (FMO) controls. THP-1 derived macrophages were stimulated with Cer, dhCer, GlcCer, LacCer, SM, S1P or PBS during 24 h. IL-6 release into the medium was measured with ELISA (BioLegend) according to the manufacturer's protocol. An additional experiment was performed to investigate the release of other inflammatory cytokines associated with Cer, dhCer, GlcCer and LacCer in the human plaque tissue. HCASMC cells were incubated in 0.1µM Cer, dhCer, LacCer and GlcCer and the release of inflammatory cytokines and growth factors (IL12p70, MCP-1, MIP-1β, TNF-α, RANTES, IL-10, PDGF) were assessed in the medium using Luminex technology.

**Caspase-3**
Cell death in plaque homogenates was analyzed using active Caspase-3 ELISA kit. The procedure was performed according to the manufacturer’s instructions (Invitrogen Caspase-3 (active) Human ELISA, Invitrogen Corporation, Camarillo, Ca, USA) and analyzed with Luminex 100 IS 2.3 (Austin, Texas, USA).

**Gene Expression**
Gene expression of enzymes (Supplemental table III) involved in sphingolipid and glycosphingolipid metabolic pathways was evaluated from global transcriptome RNAsseq data collected from 16 plaques: eight plaques not associated with symptoms and eight plaques associated with symptoms. RNA was prepared from standard total RNA extraction with Trizol cleared of Ribosomal RNA using Ribo-Zero™ Magnetic Kit from (Epicentre). Strand specific RNAsseq libraries were prepared with ScriptSeq™ v2 RNA-Seq Library v2 Preparation Kit (Epicentre). Libraries were sequenced on Illumina HiSeq2000 platform. Genomic alignments were performed with STAR and differential expression was analysed using EdgeR.\(^8,9\)

**Statistical Analysis**
Plaque contents of all the above mentioned components were normalized to the wet weight of the plaques and are expressed as median and interquartile range (IQR). All lipids were non-normally distributed. Therefore Mann-Whitney test was used for two-group comparison and Kruskal-Wallis test if more than two groups were compared. For correlations Spearman’s was done and for semi-quantitative analysis Chi-Square was performed. ANOVA followed by Bonferroni’s post hoc test were used to compare multiple groups during cell culture experiments. Values of P<0.05 were considered statistically significant. SPSS 21.0 (IBM Corp., Amonk, NY, USA) was used to perform statistical analysis.

**References:**
