Studies on the role of glycosphingolipids in metabolism

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Chapter 8

Low HDL cholesterol levels in type I Gaucher disease do not lead to an increased risk of cardiovascular disease

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Abstract

Objectives and background
A low plasma high-density lipoprotein cholesterol (HDL-c) concentration is an important risk factor for the development of atherosclerotic cardiovascular disease. HDL-c levels are abnormally low in type I Gaucher disease (GD) patients. The aim of this study was to determine whether GD is associated with premature atherosclerosis.

Methods
Lipid profiles, apolipoproteins, and carotid artery intima-media thickness (cIMT) were analyzed in 40 type I GD patients, 34 carriers and 41 control subjects. cIMT is a non-invasive validated biomarker for the status of atherosclerosis and present and future cardiovascular disease risk.

Results
Compared to control subjects, patients showed decreased HDL-c (1.1 ± 0.3 mmol/L) as well as mildly decreased low-density lipoprotein cholesterol (LDL-c) levels (2.8 ± 0.7 mmol/L), with an increased ApoB/ApoA1 ratio. In carriers, HDL-c levels were normal, but LDL-c levels were decreased (2.7 ± 0.8 mmol/L). Mean cIMT measurements were not different in the three study groups (patients: 0.63 ± 0.1mm versus carriers: 0.64 ± 0.1mm versus control subjects: 0.65 ± 0.1 mm).

Conclusions
In Gaucher disease low HDL-c levels do not lead to premature atherosclerosis as assessed by cIMT measurement. This indicates that the inverse relationship between levels of HDL-c and risk of cardiovascular disease in the general population may not be present in all conditions characterised by low HDL-c levels.
Introduction

Type 1 Gaucher disease (GD) is a lysosomal storage disorder, with a prevalence of 1:50,000 in most countries. The disorder is characterized by a deficiency of the lysosomal enzyme glucocerebrosidase, which results in accumulation of glucocerebroside in macrophages, so called Gaucher cells. Type 1 GD is the most prevalent form and can manifest itself at any age. The presence of Gaucher cells in liver, spleen and bone marrow results in hepatosplenomegaly, skeletal disease and cytopenia. In addition to these classical symptoms, GD is associated with several co-morbidities such as an increased prevalence of malignancies, hypergammaglobulinemias, pulmonary hypertension, polyneuropathies and an abnormal cholesterol profile.

In a study by Ginsberg et al low levels of total plasma cholesterol (TC), low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) in GD patients were reported. The reductions of LDL-c and HDL-c were associated with reduced levels of their respective major protein components apolipoprotein B100 (ApoB) and apolipoprotein A1 (ApoA1), indicating reduced numbers of these particles, while apolipoprotein E (apoE) levels were reported to be high. The levels of LDL-c and HDL-c were inversely correlated with parameters of disease severity and splenectomy was associated with a subsequent increase in LDL-c and HDL-c. In another study decreased levels of LDL-c and HDL-c were associated with enhanced fractional catabolism of LDL-c and HDL-c. Taken together with the increased level of apoE this suggests a central role for the macrophages in these abnormalities.

Interestingly, although carriers of a mutation in the GBA gene coding for glucocerebrosidase (carriers) do not exhibit any Gaucher symptoms, significantly lower HDL-c levels have also been found in these subjects. Type I GD can be treated with enzyme replacement therapy (ERT) in which the intravenous administration of recombinant glucocerebrosidase leads to clearance of glucocerebroside and improvement of symptoms. Treatment with ERT for 18 months causes an increase in, but not normalization of HDL-c and ApoA1 concentrations while LDL-c and apoB levels remain unchanged.

In several epidemiologic studies it has been shown that low plasma HDL-c levels are associated with increased risk of cardiovascular disease (CVD). As a consequence, GD patients as well as carriers could be considered at risk for premature atherosclerosis. In our adult
cohort of more than 70 GD patients followed for 20 years, the occurrence of cardiovascular disease does not appear to be increased. However, this is difficult to ascertain in such a small population, with relatively young patients.

A non-invasive validated biomarker for the status of atherosclerosis and present and future cardiovascular disease risk is ultrasonographically measured carotid intima-media thickness (cIMT)\textsuperscript{13,14}. To determine whether the low HDL-c levels observed in GD patients and carriers are associated with premature atherosclerosis, cIMT was analyzed in a cross sectional study in GD patients, carriers and control subjects.

**Methods**

**Study population**
Consecutive patients were recruited at the national outpatient clinic for inherited metabolic diseases at the Academic Medical Center, Amsterdam (AMC). In all patients, the diagnosis of GD was previously confirmed by enzymatic assay as well as by mutation analysis. Patients who consented to the protocol were asked to recruit family members. These family members were either blood relatives (brothers, sisters, children) or family members sharing the same household (spouses), to control for genetic and environmental influences on lipid profiles and other determinants of CVD risk as much as possible. Subjects known with hereditary dyslipidemia were excluded (n=1). Medical history of CVD (myocardial infarction (MI), stroke), the presence of cardiovascular risk factors (smoking, diabetes, hypertension), and use of alcohol and medication were surveyed by questionnaires. Blood pressure, length and weight were measured. Hypertension was defined as a blood pressure >140/90 mmHg. In addition, in GD patients severity score index (SSI, as described by Zimran)\textsuperscript{15}, use and duration of use of substrate deprivation or enzyme replacement therapy and genotype were recorded from patient files. The study was approved by the local Medical Ethical Committee and all participants provided written informed consent.

**Laboratory and genetic analysis:**
In all subjects, blood samples were drawn after an overnight (12 hour) fast. C-reactive protein (CRP) was determined. TC, HDL-c and triglycerides (TG) were measured by enzy-
matic colorimetric procedure. LDL-c was calculated by means of the Friedewald formula. ApoA1 and ApoB were determined by immunonephelometry. The concentration of apoE in plasma was determined by a turbidimetric immuno assay, as described by the manufacturer (Randox, Westburg, the Netherlands) using a Cobas Mirs auto analyzer (Roche, Basel Switzerland). Reference values were as follows: TC: 3.9-6.5 mmol/L; LDL-c: <4.49 mmol/L; HDL-c: male >1.1 mmol/L, female > 1.2 mmol/L; TG: 0.5-2.0 mmol/L; ApoA1: male 1.1-1.8 g/L, female 1.1-2.1 g/L; ApoB: male 0.55-1.4 g/L, female 0.55-1.25 g/L. In all non-Gaucher patients of whom the mutation status was unknown, mutation analysis of the GBA gene was performed. DNA was extracted from peripheral blood leukocytes and the familial GBA mutations (if applicable) and/or the six most prevalent Gaucher mutations in the Dutch population were determined (p.Asn409Ser (N370S), p.Leu483Pro (L444P), p.Arg159Trp (R120W), c.84dupG, RecNci combination (p.Leu483Pro (L444P)), p.Ala495Pro (A456P) and p.Val499Val (V460V)) and p.Leu363Pro (L324P)). Together, these six mutations account for >82% of the disease-causing alleles in the Dutch GD population. To investigate a possible relation of the HDL-c levels with Gaucher cell burden, chitotriosidase activity was measured. This enzyme, produced by Gaucher cells, indicates the total Gaucher cell burden and the plasma activity is directly related to the amount of accumulated glycolipid in spleen. Levels in carriers of the common chitotriosidase mutation were multiplied by two since heterozygosity for this mutation results in a 50% reduction in enzyme activity.

Carotid intima-media thickness
B-mode ultrasound images were acquired using an Acuson Aspen (Siemens/Acuson Corporation, Erlangen, Germany and Mountainview, CA, USA) using an L7 5-12MHz broadband transducer. Bilaterally, images of predefined arterial far wall segments of the right and left common carotid artery, the carotid bulb and the internal carotid artery were acquired. Images were saved and IMT was measured by one image analyst, blinded for the clinical and genetic status of the patient. cIMT was defined as the average of the six IMT measurements.

Statistical analysis
ApoB/ApoA1 ratios were calculated. Descriptive statistics were used for exploration of the data. Correlation between HDL-c and chitotriosidase was calculated using a Spearman test.
Differences in variables with a continuous or a dichotomous distribution between GD patients, carriers and control subjects were evaluated using linear or logistic regression analyses, respectively. These analyses were performed using the generalized estimating equations (GEE)-method in the SAS procedure GENMOD to account for correlations within families. The exchangeable correlation structure was used for these models. For differences in cIMT and the ApoB/ApoA1 ratio between the three groups, a stepwise backward multivariate regression analysis was used to adjust for potential confounders. Variables with a skewed distribution were log-transformed before statistical analyses. P-values <0.05 were considered to indicate statistical significance. A p-value of <0.10 was considered indicative of a trend. The analyses were performed with the SAS package version 9.1 (SAS Institute Inc, Cary, NC, USA).

**Results**

**General characteristics**
A total of 115 subjects (40 patients, 34 carriers and 41 control subjects) was investigated. Age, gender distribution, BMI, cigarette and alcohol use, CRP and the prevalence of hypertension were comparable in the three study groups (table 1). Three patients, four carriers and two control subjects used lipid lowering drugs. The number of subjects with a cardiovascular event in the past was also not different between groups. Thirty-four of the patients received ERT (Cerezyme, imiglucerase, Genzyme Corp., Mass., USA) for a median of 13 years (range 2-16). Two patients used substrate deprivation therapy (Miglustat, ZavescaTM, Actelion Pharmaceuticals, Switzerland), both for 9 years. The mean SSI in the patients was 8 ± 4.

**Lipids and lipoproteins**
In table 1 and figure 1, the levels of lipids and lipoproteins are shown. Levels of HDL-c and ApoA1 were significantly lower, and TG levels significantly higher, in patients as compared to control subjects (p<0.0001, p<0.0001 and p=0.01 respectively) and carriers (p<0.0001, p<0.0001 and p=0.01 resp). In patients compared to control subjects TC levels were significantly lower, while LDL-c, but not apoB levels showed a trend towards lower levels (p=0.003 and p=0.06 respectively). In carriers, no differences in HDL-c and ApoA1 levels were de-
<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Carriers</th>
<th>Control subjects</th>
<th>p patients vs control subjects</th>
<th>p patients vs carriers</th>
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<td>40</td>
<td>34</td>
<td>41</td>
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<tr>
<td>Age (years)</td>
<td>51.4 ± 11.4</td>
<td>48 ± 16.5</td>
<td>47.7 ± 16.6</td>
<td>0.22</td>
<td>0.37</td>
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<td>Sex (m/f)</td>
<td>23/17</td>
<td>Dec-22</td>
<td>19/22</td>
<td>0.65</td>
<td>0.07</td>
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<td>BMI (kg/m2)</td>
<td>24.4 ± 3.0</td>
<td>25.0 ± 2.6</td>
<td>25.1 ± 3.5</td>
<td>0.30</td>
<td>0.33</td>
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<td>Hypertension (y/n)</td>
<td>13/29</td>
<td>13/21</td>
<td>15/26</td>
<td>0.60</td>
<td>0.46</td>
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<td>CRP (mg/L)</td>
<td>2.2 ± 2.2</td>
<td>2.1 ± 1.7</td>
<td>2.3 ± 2.6</td>
<td>0.93</td>
<td>0.78</td>
<td>0.77</td>
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<tr>
<td>Alcohol (U/wk)</td>
<td>5.0 ± 6.3</td>
<td>5.8 ± 7.1</td>
<td>7.6 ± 7.7</td>
<td>0.06</td>
<td>0.47</td>
<td>0.31</td>
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<td>Smoking (y/n)</td>
<td>Jun-34</td>
<td>Sep-25</td>
<td>Dec-29</td>
<td>0.11</td>
<td>0.17</td>
<td>0.96</td>
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<td>History of MI (y/n)</td>
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<td>Mar-31</td>
<td>0/41</td>
<td>-</td>
<td>0.09</td>
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<td>History of stroke (y/n)</td>
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<td>0/41</td>
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<th>Plasma lipoprotein (mmol/L)</th>
<th>Patients</th>
<th>Carriers</th>
<th>Control subjects</th>
<th>p patients vs control subjects</th>
<th>p patients vs carriers</th>
<th>p carriers vs control subjects</th>
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<tbody>
<tr>
<td>TC</td>
<td>4.5 ± 0.9</td>
<td>4.7 ± 0.9</td>
<td>5.2 ± 1.2</td>
<td>0.003</td>
<td>0.30</td>
<td>0.07</td>
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<tr>
<td>LDL-c</td>
<td>2.8 ± 0.7</td>
<td>2.7 ± 0.8</td>
<td>3.2 ± 1.1</td>
<td>0.06</td>
<td>0.54</td>
<td>0.03</td>
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<td>HDL-c</td>
<td>1.1 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>1.6 ± 0.4</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.90</td>
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<tr>
<td>TG</td>
<td>1.0 (0.7-1.5)</td>
<td>0.7 (0.6-0.9)</td>
<td>0.8 (0.5-1.1)</td>
<td>0.01</td>
<td>0.01</td>
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<th>Plasma apolipoprotein (g/L)</th>
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<th>Carriers</th>
<th>Control subjects</th>
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<th>p patients vs carriers</th>
<th>p carriers vs control subjects</th>
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<td>ApoA1</td>
<td>1.3 ± 0.2</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.3</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.40</td>
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<td>ApoB</td>
<td>0.9 ± 0.2</td>
<td>0.8 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.64</td>
<td>0.12</td>
<td>0.07</td>
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<tr>
<td>ApoB/ApoA1 ratio</td>
<td>0.7 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td>0.01</td>
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<tr>
<td>Apo E</td>
<td>3.7 ± 1.5</td>
<td>3.4 ± 1.5</td>
<td>3.8 ± 1.2</td>
<td>0.32</td>
<td>0.69</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Table 1  Characteristics and levels of cholesterol, triglycerides and apolipoproteins of Gaucher disease patients, carriers of a glucocerebrosidase mutations and control subjects. Continuous variables are given as mean ± standard deviation, except for triglycerides which are given as median (interquartile range). Abbreviations: m=male; f=female; BMI=body mass index; y=yes; n=no; CRP=C-reactive protein; U/wk=units per week; MI=myocardial infarction; TC=total cholesterol; LDL-c=low-density lipoprotein cholesterol; HDL-c=high-density lipoprotein cholesterol; TG=triglycerides; ApoA1=apolipoprotein A1; ApoB=apolipoprotein B; ApoE=apolipoprotein E.
Figure 1  Levels of cholesterol, triglycerides, apolipoproteins and c-IMT measurements of Gaucher disease patients, carriers of a glucocerebrosidase mutations and control subjects. Abbreviations: LDL-c=low-density lipoprotein cholesterol; HDL-c=high-density lipoprotein cholesterol; ApoA1=apolipoprotein A1; ApoB=apolipoprotein B; cIMT=carotid intima media thickness.
tected in comparison to control subjects, while the trend towards a lower TC was explained by significantly decreased LDL-c levels (p=0.03), with a slighter decrease in apoB levels (p=0.07). In fact the decrease in LDL-c was more pronounced in carriers than in the GD patients. There was no significant difference between any of the groups in ApoE levels. These results did not change after correcting for gender. The ApoB/ApoA1 ratio was significantly higher in patients compared to carriers (p<0.0001) and healthy control subjects (p<0.0001) and significantly lower in carriers compared to control subjects (p=0.01). The differences in ApoB/ApoA1 ratio remained significant after multivariate analysis, adjusting for gender, age and the use of lipid lowering drugs.

**Chitotriosidase**
Of 36 Gaucher patients data on chitotriosidase activity levels, measured within 3 months of the apolipoprotein and cIMT analysis, were available. There was a weak but significant negative correlation between chitotriosidase activity and HDL-c (r=-0.35, p=0.03). All patients with a chitotriosidase level higher than 15000 nmol/ml*hour, which is considered to reflect a large Gaucher cell burden17, had a HDL-c of less than 1 mmol/L (n=6).

**Intima media thickness**
The mean cIMT was 0.63 ± 0.1mm in GD patients, compared to 0.64 ± 0.1mm and 0.65 ± 0.1 mm in the carrier and control group, respectively (figure 1). Univariate analyses revealed no differences in cIMT between the three groups. Also by multivariate analysis, after adjustment for gender and age, no significant difference between the three groups could be detected.

**Discussion**
In this study we have established that low levels of HDL-c in type I GD do not result in premature atherosclerosis as assessed by cIMT. The importance of low HDL-c levels as a risk factor for the development of atherosclerotic CVD is supported by many studies. In the Framingham Heart Study, HDL-c was a more potent risk factor for subsequent coronary artery disease than LDL-c, TC, or plasma TG11. A report combining the data from four
prospective American population studies showed that the risk for coronary artery disease decreases 1.9 to 2.9% with a 0.026 mmol/L increase in HDL-c level\textsuperscript{12}.

In the current study, as demonstrated in previous studies, low levels of HDL-c and apoA1 were reported in GD patients, as compared to healthy control subjects and carriers of a GBA mutation. Since the majority of patients in our study were treated for more than a decade, this indicates that HDL-c levels do not normalize during long term treatment. Using cIMT measurements we established that there is no indication that the low HDL-c levels in GD are associated with an increased risk for CVD. One could argue that the normal cIMT values in GD patients were the result of the relatively low levels of LDL-c, counteracting the effect of low HDL-c levels. We therefore calculated the ApoB/ApoA1 ratio which is an alternative, and in some studies superior, marker to assess CVD risk, especially in individuals with normal or low LDL-c levels\textsuperscript{19}. Comparing ApoB/ApoA1 ratios in the GD patients to the ratios calculated in a large population cohort indicates that GD patients have an odds ratio of 1.42 for future CAD based on the apolipoprotein levels in the current study\textsuperscript{20}. The odds ratio for our control group, based on comparison to these data is 1. Since short term treatment with ERT leads to elevation of ApoA1 without affecting ApoB levels\textsuperscript{10}, the ratio will have been even more unfavourable during the years prior to treatment. Patients therefore will have been exposed to an even greater CVD risk.

cIMT measurement is widely accepted as a modality that can reliably predict the risk of cardiovascular disease and as such is used as a non-invasive surrogate marker in lipid-lowering drug trials\textsuperscript{21}. In the Rotterdam study, a large cohort of healthy individuals was followed for four years registering the occurrence of stroke and myocardial infarction. At baseline cIMT was measured. In this study an odds ratio for myocardial infarction of 1.4, comparable to the odds ratio calculated in GD patients based on the ApoB/ApoA1 ratio, was associated with an 0.16 mm increase in cIMT\textsuperscript{22}. Our results show that the expected increase in cIMT in GD patients was not present, since cIMT values were the same in GD patients, carriers and healthy control subjects.

In an earlier publication Pocovi et al suggested that heterozygosity for a GBA mutation might be the most common genetic cause of low α-lipoproteinemia in the general population\textsuperscript{9}. HDL-c and ApoA1 levels were not decreased in GD carriers in the current study and mutations in the GBA gene are therefore not likely to contribute to the occurrence of hypo-α-lipoproteinemia. On the contrary, carriers were found to have low plasma LDL-c levels,
which in combination with their normal HDL-c levels, may even be considered a favorable lipid profile. However, this possible advantageous lipid profile was not reflected in a lower cIMT value, indicating no positive influence of their lipid profile on vessel wall thickening. The mechanisms resulting in low HDL-c levels in GD are only partially understood. In a study using radio-isotope labelled particles in untreated GD patients, enhanced catabolism of HDL and LDL particles was established, pointing towards the macrophages as the responsible cells. The increase in apoE in these patients may facilitate reverse cholesterol transport, resulting in increased clearance and lower levels of HDL-c. Evidence of enhanced cholesterol efflux by macrophage-derived apoE-containing lipoproteins comes from experiments showing that lipid loading of macrophages stimulates synthesis and secretion of apoE, subsequently facilitating cholesterol release from these cells. In this study however, we did not establish an increased apoE plasma concentration in GD patients. This is probably due to the effect of ERT since, in contrast to earlier studies, most of our patients were treated. In an additional analysis of sixteen patients from the cohort described in this paper, treatment with ERT for four years resulted in a significant decrease in ApoE levels (data not shown). The persistence of low HDL-c levels, despite normalization of the ApoE levels, suggests that the HDL-c decrement in GD is at least not completely dependent on ApoE mediated reverse cholesterol transport.

Both storage macrophages in Gaucher disease and foam cells in atherosclerosis are characterized by activation as a result of lipid accumulation, although the pattern of activation differs. Since Gaucher cells are not present in vessel walls, it is unlikely that vessel wall pathology is directly affected by Gaucher cells. However, the influence of circulating macrophage derived factors cannot be excluded.

Some limitations of our study warrant further discussion. The study sample was relatively small, although the earlier mentioned studies using cIMT in rare genetic diseases did show significant differences in even smaller cohorts. Furthermore, most patients had received ERT for several years, which may have influenced the levels of HDL-c and subsequently the risk for cardiovascular events, though 27 of 42 patients (64%) still had a HDL-c level below the lower limit of normal. This is consistent with a previous study showing that therapy only partially corrected the HDL-c levels.

In other genetic disorders that lead to low HDL-c levels, it has already become clear that there is not always an unequivocal relationship between the decrease in HDL-c and risk of
CVD. Different risks for the development of atherosclerosis have been established. For example, heterozygosity for an apoA-I mutation resulting in 50% loss of apoA-I concentration and a concomitant decrease in HDL-c levels, yields a more pronounced effect on atherosclerosis progression than similar reductions in HDL-c due to loss of either ABCA1 or LCAT activity (for review see 26). As a consequence, HDL-c levels per se do not necessarily reflect the atheroprotective potential of HDL-c. In type I Gaucher disease, the mechanism causing hypo-α-lipoproteinemia is most likely to be related to the degree of glycosphingolipid accumulation, since there was a negative correlation between burden of disease as measured by chiotriosidase activity and HDL-c. In addition, patients who despite treatment had a high residual burden of disease, all had HDL-c levels below 1 mmol/L.

Glycosphingolipids in plasma are present in lipoproteins (HDL, VLDL and LDL particles) and altered plasma glucosylceramide and ganglioside (GM3) levels, which have been reported in Gaucher disease27,28, may therefore change the nature and metabolic fate of these particles. In conclusion, in Gaucher disease low HDL-c levels do not lead to premature atherosclerosis as assessed by cIMT measurement, indicating that the inverse relationship between levels of HDL-c and risk of CVD in the general population may not be present in all conditions characterised by low HDL-c levels.

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