Advanced Glycation Endproducts and the Absence of Premature Atherosclerosis in Glycogen Storage Disease 1a

Nynke C. den Hollander¹, Douwe J. Mulder¹, Reindert Graaff², Suzanne R. Thorpe³, John W. Baynes³, G. Peter A. Smit⁴, Andries J. Smit¹

¹Department of Internal Medicine, University Medical Center Groningen, Groningen, The Netherlands; ²Department of Biomedical Engineering, University Medical Center Groningen, Groningen, The Netherlands; ³Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina, USA; ⁴Department of Pediatrics, University Medical Center Groningen, The Netherlands

J Inherit Metab Dis 2007; [Epub ahead of print].
Abstract

Introduction Despite their unfavorable cardiovascular risk profile, patients with Glycogen Storage Disease type 1a (GSD 1a) do not develop premature atherosclerosis. We hypothesized that this paradox may be related to a decreased formation of Advanced Glycation Endproducts (AGEs) resulting from lifetime low plasma glucose levels and decreased oxidative stress.

Methods In 8 GSD 1a patients (age 20-34 yrs) and 30 matched controls we measured carotid intima media thickness (IMT), skin autofluorescence (AF), a non-invasive index for AGEs, and specific AGEs (pentosidine, N-(carboxymethyl)lysine (CML), N-(carboxyethyl)lysine (CEL)) and Collagen Linked Fluorescence (CLF, measured at excitation/emission wavelength combinations of 328/378 and 370/440 nm) in skin samples.

Results Carotid IMT was significantly lower in GSD 1a patients. Skin AF did not differ between patients and controls. The skin samples showed higher CEL levels in the patient group (p=0.008), but similar levels of pentosidine, CML, and CLF. In the total group, skin AF correlated with CML (r=0.39, p=0.031), and CLF 328/378 nm (r=0.53; p=0.002) and CLF 370/440 nm (r=0.60; p=0.001). In the control group, AF also correlated with the maximum carotid IMT (r=0.6; p=0.004).

Conclusion Although our data confirm that GSD 1a patients present with a reduced burden of atherosclerosis this phenomenon cannot be explained by differences in AGE accumulation as measured in the skin.
Introduction

Glycogen Storage Disease type 1a (GSD 1a, von Gierke disease; OMIM +232200) is an inborn error of metabolism, caused by a deficiency of microsomal glucose-6-phosphatase (G6Pase; EC 3.1.3.9) in the liver and kidneys. G6Pase is a key enzyme in the homeostatic regulation of blood glucose concentration by catalyzing the terminal step in both glycogenolysis and gluconeogenesis.

Although the main feature of this disease is severe fasting hypoglycemia, several other metabolic changes complicate the disease. GSD 1a patients have marked hyperlipidemia, characterized by a high total cholesterol with a low High Density Lipoprotein (HDL) and, most strikingly, high triglyceride levels, a result of both increased synthesis and, possibly, decreased plasma lipid clearance. Another long-term complication is renal failure. After a silent period of hyperfiltration, patients develop micro-albuminuria and eventually frank proteinuria during the first three decades of life. Dyslipidaemia and microalbuminuria are known to be strong risk factors for atherosclerosis and cardiac disease1,2. As patients are surviving longer, concern has arisen that GSD 1a patients may be at high risk for atherosclerosis as well3. However, in contrast to other diseases associated with exposure to cardiovascular risk factors at an early age, such as Familial Hypercholesterolaemia4, it has been shown that GSD 1a patients do not show any signs of endothelial dysfunction or premature atherosclerosis5-7.

Recently, the accumulation of Advanced Glycation Endproducts (AGEs) on tissue proteins has been implicated as a contributing factor in atherosclerosis8-11. AGEs are a heterogeneous group of compounds formed by the Maillard reaction, or the non-enzymatic browning reaction that modifies proteins with carbohydrate- and lipid-derived intermediates12. AGE formation is increased in hyperglycemia and under the influence of oxidative stress13-17. AGE formation alters the characteristics of both short- and long-lived (eg. collagen) proteins. AGEs accumulate in extra cellular matrix proteins, but also in atherosclerotic plaques18;19 and the expression of receptors for AGEs (RAGE) is increased in atherosclerosis20. Interaction of AGE-modified proteins with AGE receptors may stimulate cytokine and growth factor production that sustains the development of the atherosclerotic plaques.

Therefore, we postulated that as increased AGE formation leads to atherosclerosis, decreased AGE formation might protect against this disease. We hypothesized that GSD 1a patients have lower AGE levels based on two observations. First, GSD 1a patients may more frequently be hypoglycemic21;22. Second and most importantly, GSD 1a patients have significantly lower levels of oxidative stress23. Low AGE levels in women with polycystic ovary syndrome, a common complication in GSD 1a24 that also increases the risk for atherosclerosis, also contributes to this hypothesis25.

In the current study, we assessed AGEs in tissue both by quantification of specific AGEs and measurement of collagen linked fluorescence (CLF) in skin biopsies, and by measuring skin autofluorescence (AF), using a recently developed and validated method(26). We aimed to investigate whether patients with GSD 1a present with lower levels of AGE accumulation
compared with age and sex matched healthy controls.

Methods

Subjects
A total of 38 adults were enrolled in this study, 8 GSD patients (2 male and 6 female) age 18–36 (27 ± 7) yr and 30 healthy control subjects matched for age and sex (8 male and 22 female). All GSD patients aged ≥ 18 years, who attended our outpatient clinic or were admitted during the study period, were approached for participation and age-matched controls were approached after the selection of individual patients. All participants were assessed with the procedures described below during the same study period. Relevant information on medical history and smoking habits was obtained from both patients and control subjects. The study was approved by the local Medical Ethics Committee and written informed consent was obtained from all participants.

Skin autofluorescence (AF)
Skin AF was assessed on the ventral site of the lower arm with a prototype of the current AGE-Reader (DiagnOptics BV, Groningen, The Netherlands) as described elsewhere. In short, the AGE-Reader consists of a tabletop box, containing a black light excitation light source (peak wavelength ~360 nm). Light emitted from the skin is measured with an integrated spectrometer. Measurement is fully automated and takes approximately 30 seconds to be performed, giving an average value over 50 individual scans. Skin AF is calculated by dividing the mean value of the emitted light intensity per nm between 420-600 nm by the mean value of the excitation light intensity per nm between 300-420 nm, expressed as arbitrary units (AU). The intra-individual Altman error percentage is 5.0% on a single day and 5.9% for seasonal changes.

Carotid Intima-Media Thickness (IMT) measurement
IMT of the carotid arteries was measured by ultrasonography in the supine position. High-resolution B-mode ultrasound images (ACUSON 128 XP; Acuson Corp, Mountain View, CA) with a 7.0-MHz linear array transducer were used to measure intima-media wall thickness, as described previously in more detail. In short, the right and left common carotid arterial wall segments were imaged from a fixed lateral transducer angle at the far wall of the distal 1-cm segments of both common carotid arteries (CCA), proximal to the carotid bulb. The far wall of the carotid bulb (CB), and of the most proximal part of the internal carotid artery (ICA) were also scanned bilaterally. The scans were recorded on S-VHS tape and analyzed offline by an independent image analyst unaware of subject characteristics. B-mode image analyses were digitized with a frame grabber (DT286 I; Data Translation, Marlboro, MA). The image analysis software was developed using an algorithm developed by Selzer et al. The average and maximum carotid IMT over the six segments (CCA, CB and ICA, bilaterally far wall) of both carotid arteries were calculated. At a mean IMT of 0.80 mm, intersonographer variability amounted to 0.05 mm, with an image analyst variability of <0.03 mm, corresponding to a total variation coefficient of between 6.3 and 7.3%.
**Blood and urine collection**

Venous blood and a portion of urine were taken non-fasting to measure glycated hemoglobin A1c (HbA1c), lipid spectrum, creatinine and urine albumin, using routine laboratory methods.

**Skin samples and measurement of AGES**

A 4 mm diameter full-thickness skin biopsy was taken under 2% lidocaine-adrenaline anesthesia from the volar (frontal) side of the lower arm. The sample was snap frozen in liquid nitrogen and stored at -80 ºC. After thawing, insoluble collagen was extracted as previously described\textsuperscript{27;32}, the samples were reduced with sodiumborohydride and solubilized using pepsin. Then Collagen Linked Fluorescence (CLF) was measured using wavelengths of 328/378 nm and 370/440 nm (excitation/ emission). Both CLF and pentosidine were normalized to the hydroxyproline content of the sample according to Stegemann and Stalder\textsuperscript{33}. Pentosidine was measured by high performance liquid chromatography as described by Dyer et al.\textsuperscript{34}. N-(carboxymethyl)lysine (CML) and N-(carboxyethyl)lysine (CEL) analysis was performed by gas chromatography-mass spectrometry as described by Dunn et al.\textsuperscript{35}. CML and CEL were normalized to the lysine content of the sample.

**Statistical analysis**

Due to the low prevalence of the disease, we could not include more than 8 adult patients with GSD 1a for this study. We determined that at this given number of patients and an expected standard deviation of 0.25, a sample size of 30 controls would be needed to give the study an 80% power at $\alpha = 0.05$ to detect at least a difference of 20% between both groups.

Because of the small group measured, data are given as medians (interquartile range). The Mann-Whitney test was performed to compare groups and Spearman’s rho test to calculate correlations. All analyses were performed with SPSS version 13.0 for Windows (SPSS, Chicago, IL, U.S.A.). $p<0.05$ was considered statistically significant.

**Results**

One control subject was excluded because micro-albuminuria appeared to be present. The results of the skin biopsy measurements from two male subjects were excluded because the amount of material was not sufficient to get reliable data, based on hydroxyproline. No sex-based differences were present.

All patients used a xanthine-oxidase inhibitor and 7 out of 8 patients also used an Angiotensin-converting Enzyme inhibitor. The observed hyperlipidemia was not treated in any of the patients. Other characteristics of both patient and control group are listed in Table 1. It shows that the GSD patients enrolled in this study had similar levels of HbA1c. The patients were markedly dyslipidemic with higher triglycerides, total cholesterol and lower HDL cholesterol compared to controls. The maximum carotid IMT and the average carotid IMT in patients were significantly lower than in the control group.
Table 1. Characteristics of patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 29)</th>
<th>Patients (n = 8)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>25 (21-32)</td>
<td>27 (20-34)</td>
<td>NS</td>
</tr>
<tr>
<td>Length (m)</td>
<td>1.78 (1.69-1.85)</td>
<td>1.66 (1.61-1.68)</td>
<td>0.019</td>
</tr>
<tr>
<td>Sex (male/ female)</td>
<td>8/21</td>
<td>2/6</td>
<td>NS</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>23 (21-26)</td>
<td>24 (22-28)</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>1</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.9 (4.6-5.0)</td>
<td>4.8 (4.6-5.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerids (mmol/l)</td>
<td>1.19 (0.88-1.63)</td>
<td>4.05 (3.07-7.10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.90 (4.15-5.35)</td>
<td>5.88 (5.22-7.72)</td>
<td>0.012</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.44 (1.32-1.76)</td>
<td>1.14 (1.00-1.25)</td>
<td>0.008</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>2.53 (2.10-3.04)</td>
<td>3.07 (1.61-1.68)</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (umol/l)</td>
<td>81 (75-92)</td>
<td>66 (65-73)</td>
<td>0.002</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)†</td>
<td>125 (112-142)</td>
<td>143 (123-153)</td>
<td>0.086</td>
</tr>
<tr>
<td>Albumin/creatinine ratio</td>
<td>0.8 (0.5-1.2)</td>
<td>1.2 (0.6-6.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Average carotid IMT (mm)</td>
<td>0.60 (0.58-0.62)*</td>
<td>0.53 (0.48-0.59)</td>
<td>0.008</td>
</tr>
<tr>
<td>Maximum carotid IMT (mm)</td>
<td>0.77 (0.74-0.81)*</td>
<td>0.68 (0.60-0.74)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data are given as median and interquartile range or as absolute numbers. Differences between groups are calculated with the Mann-Whitney U test or Chi-square test where appropriate.

* n=27; † Creatinine clearance as calculated by the Cockcroft-Gault formula; IMT indicates Intima Media Thickness; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein; HbA1c, glycated hemoglobin A1c, NS, not significant.

Skin AGEs, measured in skin samples
Table 2 demonstrates that skin samples of patient versus control subjects showed comparable amounts of the AGEs pentosidine and CML. CEL levels were significantly higher in the patient group (see Figure 1). No differences were found between patients and controls in CLF 328/378 or CLF 370/440. Skin AF did not show any differences between patients and controls (see Figure 2).

Correlations between indices for AGE accumulation and IMT
In both patients and controls, skin AF correlated with CLF 370/440 (r=0.62; p<0.001) and also with CML (r=0.39; p=0.031) and CLF 328/278 (r=0.53; p=0.002). Skin AF did not correlate with pentosidine or CEL. All AGEs measured from skin biopsies correlated significantly with each other, apart from CEL which did not correlate with pentosidine.

In the control group, skin AF correlated with maximum carotid IMT (r=0.60; p=0.004, see Figure 3), but not with mean carotid IMT (r=0.33; p=0.139). Also, in both patients and controls there was no correlation between carotid IMT and skin AF.
Table 2. Characteristics of patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 29)</th>
<th>Patients (n = 8)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autofluorescence (* 10^-2 AU)</td>
<td>1.55 (1.30 - 1.76)</td>
<td>1.67 (1.57 - 1.76)</td>
<td>NS</td>
</tr>
<tr>
<td>Pentosidine (ug/g hydroxyproline)</td>
<td>23.8 (18.0-30.6)†</td>
<td>19.1 (16.7-28.4)*</td>
<td>NS</td>
</tr>
<tr>
<td>CML (mmol/mol lysine)</td>
<td>1.01 (0.81-1.47)†</td>
<td>1.14 (0.78-1.59)*</td>
<td>NS</td>
</tr>
<tr>
<td>CEL (mmol/mol lysine)</td>
<td>0.05 (0.05-0.07)†</td>
<td>0.09 (0.05-0.13)*</td>
<td>0.008</td>
</tr>
<tr>
<td>CLF 328/378 (AU/ug hydroxyproline)</td>
<td>324 (239-388)</td>
<td>318 (286-374)*</td>
<td>NS</td>
</tr>
<tr>
<td>CLF 370/440 (AU/ug hydroxyproline)</td>
<td>203 (163-259)</td>
<td>197 (179-324)*</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are given as median and interquartile range and differences between groups are calculated with the Mann-Whitney U test.

* n=7 † n=27; CML indicates N-(carboxymethyl)lysine; CEL, N-(carboxyethyl)lysine; CLF, Collagen Linked Fluorescence; AU, Arbitrary Units, NS, not significant.

Figure 1. The amount of N-(carboxyethyl)lysine (CEL) in skin samples of the patients and controls. P-value was calculated with the Mann-Whitney U test.

Figure 2. Skin autofluorescence (AF) in patients and controls. P-value was calculated with the Mann-Whitney U test. AU indicates arbitrary units.

Figure 3. The correlation between skin autofluorescence (AF) and maximum carotid intima media thickness (IMT) in control subjects only.
Discussion

In the current study we confirm the previous observation (36) that patients with GSD 1a have a thinner IMT than healthy controls. This supports the finding that these patients, despite severe hyperlipidemia and microalbuminuria, do not develop premature atherosclerosis.

Since GSD 1a patients have lower levels of whole body oxidative stress\textsuperscript{37} and a strong tendency to develop hypoglycemia\textsuperscript{38}, we hypothesized that decreased formation of AGEs might protect them from developing premature atherosclerosis. The data from this study do not support this hypothesis. GSD 1a patients did not have lower skin levels of AGEs, as measured either non-invasively using skin AF or \textit{ex vivo} in skin samples.

Our data confirm the clinical usefulness of skin AF as a tool for measuring AGE accumulation in the skin, since skin AF correlated strongly with the important representative of the AGEs, CML, and with CLF in skin biopsies. Additionally, skin AF correlated with maximum carotid IMT in healthy controls, suggesting it may be a non-invasive index to assess cardiovascular risk in apparently healthy subjects. The measurement of skin AF with the AGE-reader is a novel procedure and it may prove to be a useful tool, since the measurement of AGEs in skin samples has several obvious limitations, including its invasive nature. The AGE-reader has been validated in diabetic patients, in hemodialysis patients and healthy controls\textsuperscript{27,39}. In these validation studies AF correlated with the AGEs pentosidine, CML and CEL. Additionally, skin AF was related to clinical progression of atherosclerotic disease, since it predicted the incidence of future cardiovascular morbidity and mortality in both patient groups\textsuperscript{40}. In the current study, we confirm that skin AF correlates with CML and CLF. However it did not correlate with CEL and pentosidine. A potential explanation might be a combination of the small sample size and the narrow and lower range of CEL and pentosidine in the current young group with a small age range compared to previous studies.

Skin biopsies are a generally well accepted standard for quantifying AGE accumulation in humans. Through this method, we found that only CEL is different in GSD 1a patients. Pentosidine, the AGE that is exclusively derived from carbohydrates, does not differ between patient and control groups. CML and CEL are both carbohydrate and lipid derived. The hyperlipidemia in GSD 1a patients might therefore explain the elevated CEL levels in skin biopsies. Our data also show a correlation between triglyceride levels and CEL (data not shown). However, the normal CML levels can not be explained by this theory. AGE-formation is increased in hyperglycemia and during oxidative stress. As expected from current treatment strategies, HbA1c levels in the GSD patients are normal and comparable to those of the controls, which make important differences in glycemic stress less likely.

In diabetes elevated AGE levels in skin collagen are strongly associated with long-term macro- and microvascular complications\textsuperscript{41}. Normal (or even partially elevated) AGEs in GSD 1a patients are not of any help to explain the absence of atherosclerosis. Wittenstein \textit{et al}. compared plasma antioxidants in pediatric patients with GSD 1a, Diabetes Mellitus and hypercholesterolemia to clarify the absence of atherosclerosis\textsuperscript{42}. GSD 1a patients in their
study had increased antioxidative defense in plasma, as measured by total radical-trapping antioxidant parameters. However, these might have been strongly influenced by elevated uric acid levels in GSD 1a, which may be protective against atherosclerosis\(^43\). In our patient group, all patients used the xanthine-oxidase inhibitor Allopurinol to reduce uric acid levels in order to prevent the development of gout, which by itself may also reduce oxidative stress and has been shown to improve endothelial dysfunction\(^44;45\). These factors may have blurred the results from our study.

We found little difference in AGE levels between GSD 1a patients and controls. The extent of renal disease in GSD 1a also has to be taken into account to understand this result, since AGE accumulation is dependent on renal function\(^46\). The contribution of the risk factor microalbuminuria is attenuated, because almost all patients used an ACE-inhibitor. In our study, the albumin/creatinine ratio in patients did not differ from the ratio in the controls. The GSD 1a patients in this study did have a significantly lower creatinine, although the creatinine clearance calculated by the Cockcroft-Gault formula did not show any differences from controls. Kidney disease in GSD 1a is known to start with a period of silent hyperfiltration\(^47\)-\(^49\). During this period of hyperfiltration serum AGE levels may be lower, but whether this may have influenced skin AGEs is uncertain. AGEs linked to skin collagen are generally assumed to be an adequate reflection of overall AGE status because of the long lifespan of this protein. Measurement of AGEs in kidney and liver might reveal aberrant levels of AGEs, because glucose-6-phosphate and glycogen accumulate are known to accumulate in the liver. Glucose-6-phosphate has a higher potential of forming AGEs than glucose, so AGEs in kidney- and liver-tissue might be increased in GSD 1a patients. Measurement of AGEs in liver, kidney, and urine might help to understand the role of AGEs in GSD 1a patients. In support of this, high levels of detoxification products of the Maillard reaction have been found in a patient with glycogen accumulation in renal tubular cells comparable to GSD 1a\(^50\).

In conclusion, our study confirms that patients with GSD 1a have a slower progression of atherosclerosis compared with healthy, age matched controls. Since GSD 1a is characterized by hypoglycemia and previous studies have shown decreased levels of whole body excess of reactive oxygen species as measured by specific oxidative stress markers in the patients, we hypothesized that this phenomena could be explained by decreased formation of AGEs. However, in the present study, AGE levels, as measured non-invasively by skin AF and \textit{ex vivo} in skin biopsies, did not differ between patients and controls. Hence, the present results do not clarify why patients with GSD 1a avoid early atherosclerosis, as would be expected by their adverse cardiovascular risk profile, and suggest that other mechanisms may be involved in the preserved vascular health of these patients.
diagnosis, management, clinical course and outcome. Results of the European Study on Glycogen Storage Disease Type I (ESGSD I). Eur J Pediatr 2002; 161 Suppl 1:S20-S34.


