Discovery and characterization of new glucosylated metabolites: pathophysiological consequences

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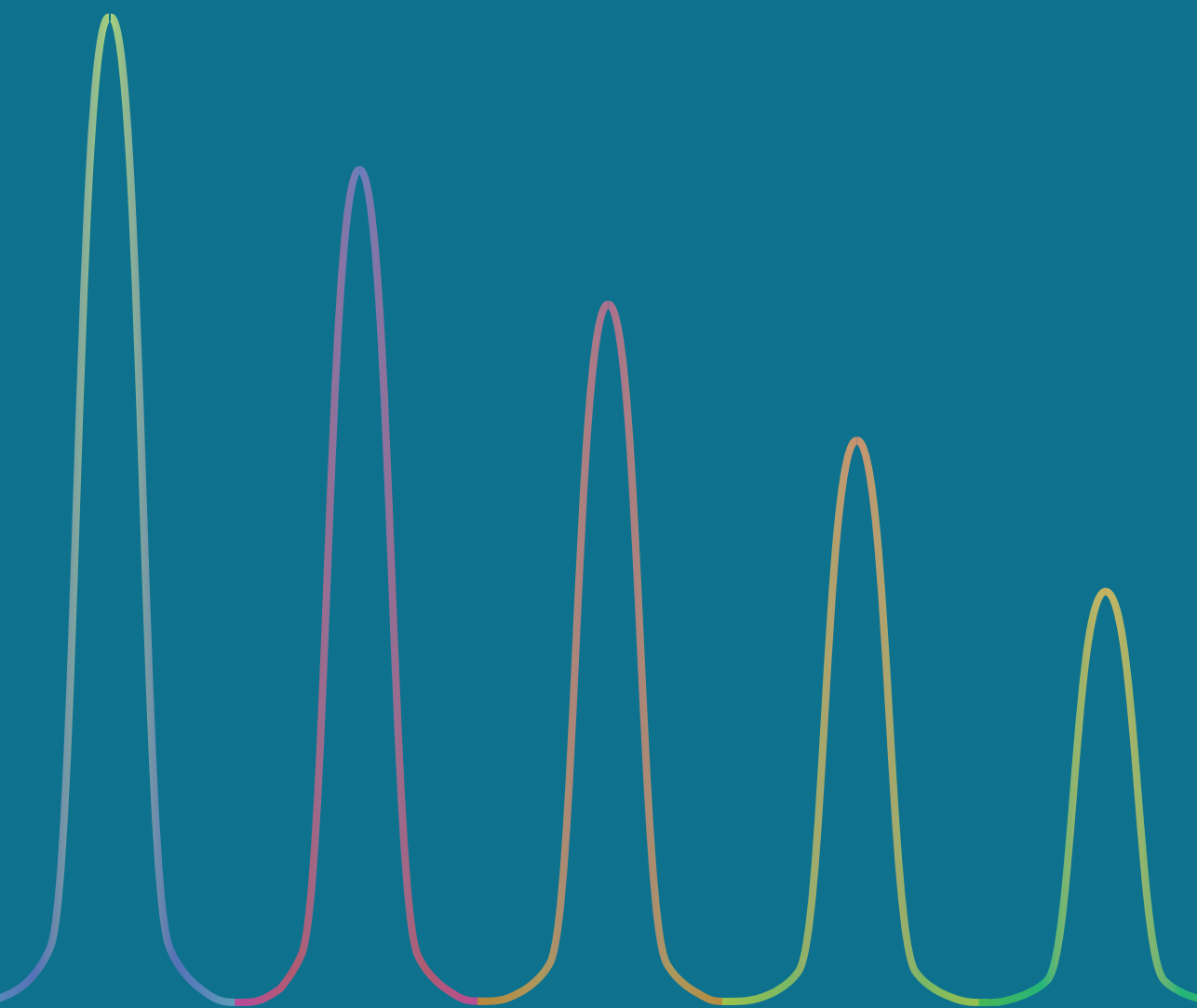

Chapter 4

Formation, degradation and natural occurrence of Glucosyl-7-dehydrocholesterol and Glucosylated vitamin D_3

To be incorporated in revised form in an invited review

Chapter 4 - Formation, degradation and natural occurrence of Glucosyl-7-dehydrocholesterol and Glucosylated vitamin D_3

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To be incorporated in revised form in an invited review

Contributions:
H.N.J.M. : author, performed described experimental biochemistry and analytical chemistry
P.W. : (BioSyn, LIC); synthesis of (isotope labeled) GlcChol, Glc7DHC and $GlcD_3$
K.K. : synthesis of (isotope labeled) GlcChol and GlcDesm
M.M. : advise with LC-MS/MS
M.J.F. : advise with sample preparation
D.E.C.B. : collaboration on extraction and measurements of metabolites in skin
C.M.B. : (LACDR); collection full skin samples
M.V. : repeated time and pH curve of *in vitro* formation of $GlcD_3$
M.A. : co-supervision
J.M.A. : supervision

Abstract

Recently the existence of additional substrates for the enzyme glucocerebrosidase (GBA) beyond glucosylceramide (GlcCer) was recognized. The discovery of glucosylated cholesterol (GlcChol) being a good substrate for GBA prompted us to study metabolites with a structural resemblance to cholesterol. We investigated, with our sensitive quantitative LC-MS/MS method, the *in vitro* formation and degradation of glucosylated 7-dehydrocholesterol (Glc7DHC). 7DHC can be converted to cholecalciferol (vitamin D_3, D_3) under influence of UVB-irradiation. Our results show that the retaining β-glucosidases GBA and GBA2 are able to transglucosylate both 7DHC and D_3 *in vitro*. However, the activity of GBA is far more prominent. Degradation by GBA was observed for Glc7DHC, degradation by GBA2 was not detected. Both β-glucosidases were found unable to degrade $GlcD_3$. We observed that the process of conversion by UVB also applies for Glc7DHC, which is converted $GlcD_3$. The natural occurrence of Glc7DHC in healthy spleens was observed. In spleens from GD patients, deficient in GBA, the levels of Glc7DHC were not significantly increased. This suggests that *in vivo* GBA is responsible for degradation of Glc7DHC. To conclude, we report the existence of two novel metabolites, the transglucosylation products Glc7DHC and $GlcD_3$.

Introduction

7-dehydrochlesterol (7DHC), is a key metabolite in two pathways: *de novo* cholesterol synthesis and vitamin D_3 (cholecalciferol, D_3) pathway (Figure 1). Within the major pathway for cholesterol synthesis, the Kandutsch-Russel pathway, 7DHC is the last precursor before cholesterol (Chol) is synthesized [1]. The enzyme Sterol C5-desaturase (SC5D, EC 1.14.19.20) converses lathosterol into 7DHC. Followed up by the action of the enzyme 7-Dehydrocholesterol Reductase (DHCR7, EC 1.3.1.21), which converses 7DHC into Chol [1-3]. In the vitamin D_3 pathway, 7DHC is converted into pre-cholecalciferol ($PreD_3$), from which via cholecalciferol (D_3) the active vitamin D_3 ($25(OH)D_3$, 25-hydroxyvitamin D_3, 25-hydroxycholecalciferol) and 1α-25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, calcitriol) is formed (Figure 1). That both pathways are entwined, comes forth from the knowledge that high cholesterol levels promote the use of 7DHC in the biosynthetic vitamin D_3 pathway [4-6].

This research focusses on 7DHC and its derivative D_3. In the skin 7DHC is converted into $PreD_3$ under influence of UVB irradiation (305 nm). A thermal dependent rearrangement of the double bonds of $PreD_3$ results in the formation of D_3. After a longer UVB exposure time, two additional photoproducts of $PreD_3$, lumisterol and tachysterol, develop by which excessive $PreD_3$ levels are prevented. Vitamin D-binding protein (DBP) translocates D_3 into the circulation, a process that positively influences the equilibrium of $PreD_3 \leftrightarrows D_3$, to assure efficient conversion to D_3 [7, 8]. When inside the bloodstream D_3 travels to the liver for enzymatic processing by 25-hydroxylase (25-OHase, CYP2R1, EC 1.14.14.24). This enzyme metabolizes D_3 into 25-hydroxycholecalciferol ($25(OH)D_3$) [9-12]. This conversion cannot only be performed by CYP2R1, but also by CYP27A1 (EC 1.14.15.15) [12, 13]. Via the bloodstream $25(OH)D_3$ is transported to the kidney, where it is converted to calcitriol ($1,25(OH)_2D_3$, active hormonal D_3) by 1α-hydroxylase (1α-OHase, CYP27B1, EC 1.14.15.18) [14]. Calcitriol fulfils several important functions. In the kidney $1,25(OH)_2D_3$ is involved in calcium and phosphate homeostasis [15-17]. Furthermore $1,25(OH)_2D_3$, but also D_3 and $25(OH)D_3$, reach other target tissues via the bloodstream, such as the intestine, bone and the parathyroid gland [18, 19]. The pathway of 7DHC to $1,25(OH)_2D_3$, not only initiates in the skin, but may also takes place in the skin completely [20-22]. In keratinocytes, $1,25(OH)_2D_3$ is involved in the regulation of calcium homeostasis [23] and it protects the skin against UVB radiation [24-27]. In addition, $1,25(OH)_2D_3$ in particular plays a key role in regulation of bone density via its interaction with the Vitamin D Receptor (VDR). The VDR is part of the steroid-thyroid hormone receptor family and mediates vitamin D target gene transcription, via the vitamin D-responsive elements (VDREs) [28-31]. One of the genes regulated via VDR and $1,25(OH)_2D_3$ is the osteocalcin gene [28-31], an important gene for bone development [32-34]. Deficiencies in D_3 and $1,25(OH)_2D_3$ are linked to vitamin-D-dependent rickets [35, 36], osteomalacia and osteoporosis [37-42]. Over the years levels of D_3 and $1,25(OH)_2D_3$ have been associated with several ailments.

For example, parathyroid-related disorders are known to be related to levels of $1,25(OH)_2D_3$ [43, 44]. But also associations with chronic obstructive pulmonary disease (COPD) [45-50], obesity [51-53], multiple sclerosis [54-56] and chronic kidney disease [57-60] have been observed.

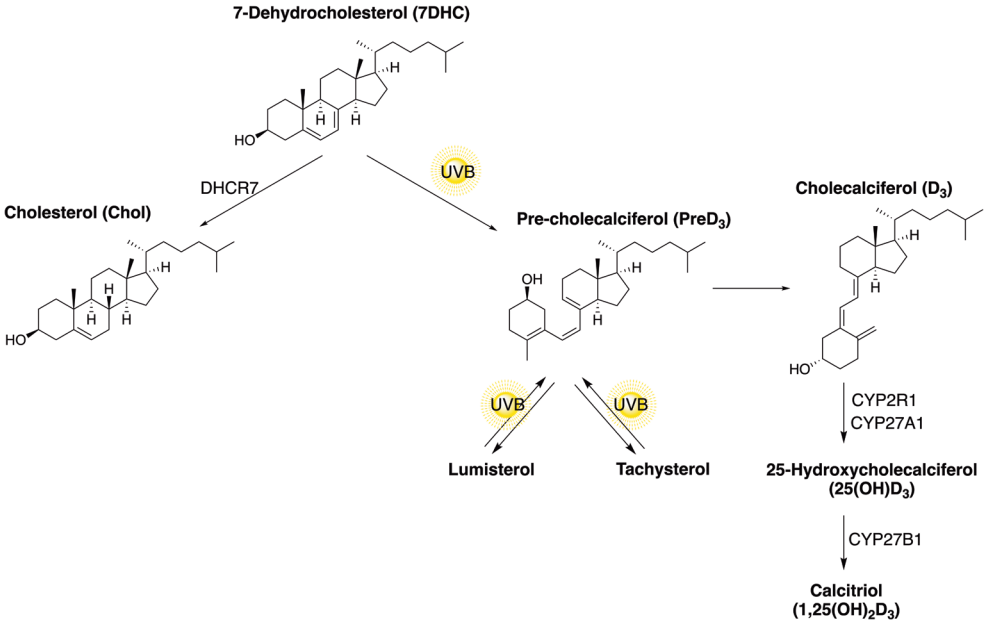


Figure 1. The fate of 7-dehydrocholesterol (7DHC). *Showing involvement of 7DHC in both the cholesterol pathway and the vitamin D_3-pathway. Cholesterol pathway: 7DHC is conversed into cholesterol (Chol) by the enzyme DHCR7 (EC 1.3.1.21). Vitamin D_3-pathway: the first reaction steps from 7DHC to D_3 occur under influence of UVB (305 nm) and 37°C. Further involved enzymes are 25-hydroxylase (25-OHase, CYP2R1, EC 1.14.14.24), 27-hydroxylase (CYP27A1, EC 1.14.15.15) and 1α-hydroxylase (1α-OHase, CYP27B1, EC 1.14.15.18).*

7DHC has also been related to disease, in particular lathosterolosis (OMIM #607330) and Smith-Lemli-Opitz syndrome (SLOS, OMIM #270400). In lathosterolosis, levels of 7DHC are decreased due to a defect in SC5D, preventing the conversion of lathosterol into 7DHC [61-63]. In SLOS, on the other hand, the level of 7DHC is increased, as the enzyme DHCR7 is defect which causes storage of 7DHC and a decrease in Chol levels [64-68]. Even though in both diseases the levels of 7DHC are opposite to one another, levels of Chol are in both diseases decreased. Consequently, there is some resemblance in the clinical manifestations of both diseases. Both diseases display multiple congenital malformations, mental retardation and developmental delay [63, 64, 69, 70].

Cholesterol is a known acceptor for transglucosylation with GlcCer. A reaction performed *in vitro* by glucocerebrosidase (GBA), the enzyme deficient in GD. *In vivo* GBA largely degrades GlcChol in lysosomes [71]. Gaucher disease (GD) is a lysosomal storage disorder presenting with a wide variety of symptoms ranging from hepatosplenomegaly, pancytopenia, coagulation abnormalities, skeletal complications, neurodegeneration and disturbances in skin permeability [72-75]. Inherited defects in the enzyme glucocerebrosidase (GBA) are the cause of GD [74, 76]. GBA hydrolyses in lysosomes the ubiquitous glycosphingolipid glucosylceramide (GlcCer) into glucose (Glc) and ceramide (Cer) [77]. As result of an inherited GBA deficiency in Gaucher patients, GlcCer stores in lysosomes, particularly of tissue macrophages that transform into characteristic Gaucher cells [72, 77, 78]. Historically, different Gaucher phenotypes are discerned based on age of onset and neurological involvement [79]. Type 1 is the most common non-neuronopathic variant, type 2 is the acute neuronopathic variant and type 3 the sub-acute neuronopathic variant. At present, only type 1 GD is effectively treated by means of enzyme replacement therapy (ERT), based on chronic bi-weekly intravenous administration of macrophage-targeted recombinant GBA, and by means of substrate reaction therapy (SRT) based on daily oral administration of an inhibitor of glucosylceramide synthase [75, 79-81]. The pathophysiology of GD is in many aspects still puzzling. The presence of Gaucher cells may explain the hepatosplenomegaly in GD patients but offers no simple explanation for their bone disease or other symptoms. In addition, poorly understood still is the heterogeneous disease manifestation among GD patients with similar GBA genotypes. Most striking in this respect are monozygotic twins with different disease severity [82, 83]. The recent recognition of the existence of additional substrates for GBA beyond GlcCer, in particular glucosylated cholesterol (GlcChol), might be relevant in this respect [71].

It is conceivable that additional glucosylated metabolites occur in GD patients and that abnormalities in these play a crucial role in onset of specific symptoms. Along this line of reasoning, we have searched for glucosylated 7-dehydrocholesterol (7DHC) and its derivative vitamin D_3 (D_3). We here show that 7DHC and D_3, like Chol, can be transglucosylated by GBA *in vitro* to Glc7DHC and $GlcD_3$. The enzyme also degrades Glc7DHC and $GlcD_3$. While GlcChol is abnormally high in GD patients, Glc7DHC levels in GD spleen are surprisingly normal.

Results

In vitro formation and degradation of Glc7DHC by transglucosylation by β-glucosidases

We first studied the ability of pure recombinant GBA to form Glc7DHC. For this the enzyme was incubated for 1 hour in 150 mM McIlvain buffer (pH 5.2), supplemented with Triton X-100 (0.1% v/v), sodium taurocholate (0.2% w/v), bovine serum albumin (0.1% w/v), 4MUGlc (3.7 mM) as glucose donor and 7DHC (0.3 mM) or Chol (0.3 mM) as acceptors. Formation of glucosylated 7DHC and Chol was monitored by LC-MS/MS.

rGBA was found to produce Glc7DHC and GlcChol over time (Figure 2A). We replaced 4MUGlc by GlcCer as potential glucose donor and observed similar formation of Glc7DHC and GlcChol (Figure 2B). Optimal pH for the generation of the glucosylated metabolites was determined and found to be between pH 4.5 and pH 5.0, coinciding with the pH of lysosomes in which GBA normally resides.

Subsequently, we investigated whether the cytosol-facing GBA2 is also able to generate Glc7DHC. For this, we incubated homogenates of cells overexpressing GBA2 with 7DHC and 4MUGlc or GlcCer as glucose donors and analysed formation of Glc7DHC. To exclude action of GBA, the homogenates were pre-treated with an irreversible inhibitor of the enzyme, adamantyl-cyclophellitol (ME656). Optimal formation of Glc7DHC by GBA2 in the homogenates was observed at slightly higher pH as for rGBA (Figure 2C, D). When homogenates were incubated with 7DHC without additional glucose donor (4MUGlc or GlcCer) hardly any Glc7DHC was detected (not shown). Of note, the *in vitro* formation of Glc7DHC by GBA2 was relatively poor, certainly in comparison of the ability of the same enzyme to form GlcChol.

Finally, we investigated the broad-specific beta-glucosidase GBA3. Previous work showed that GBA3 is not able to perform the transglucosylation reaction for GlcChol formation [71]. We neither observed detectable transglucosylation of Chol to GlcChol, or transglucosylation of 7DHC to Glc7DHC (data not shown).

To reach insight in the relative transglucosylation and hydrolase activities, we repeated the experiments above at optimal pH for GBA (pH 5.2) as well as GBA2-enriched homogenate (pH 5.8) and measured besides Glc7DHC and GlcChol also the formed 4-methylumbelliferone (4MU) (Figure 3). The formation of both products remained constant over time. Compared to rGBA, GBA2 shows a lower GlcChol formation, and no preference for Glc7DHC formation at all.

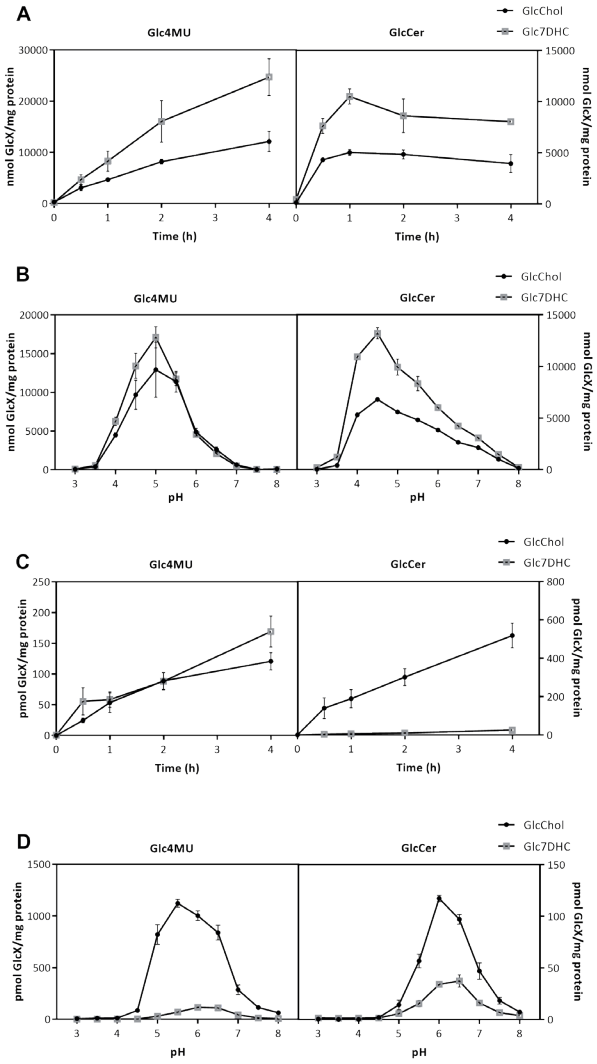


Figure 2. In vitro formation of Glc7DHC. *A) rGBA was incubated for varied times with cholesterol (Chol) or 7-dehydrocholesterol (7DHC) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer) donor. Formation of GlcChol and Glc7DHC was measured by LC-MS/MS (nmol/mg protein). B) rGBA was incubated at varied pH with Chol or 7DHC in the presence of 4MUGlc or GlcCer donor. Formation of GlcChol and Glc7DHC was measured by LC-MS/MS (nmol/mg protein). C) Lysates of cells overexpressing GBA2 were incubated for varied times with Chol or 7DHC in the presence of 4MUGlc or GlcCer donor. Formation of GlcChol and Glc7DHC was measured by LC-MS/MS (pmol/mg protein). D) Lysates of cells overexpressing GBA2 were incubated at varied pH with Chol or 7DHC in the presence of 4MUGlc or GlcCer donor. Formation of GlcChol and Glc7DHC was measured by LC-MS/MS (pmol/mg protein).*

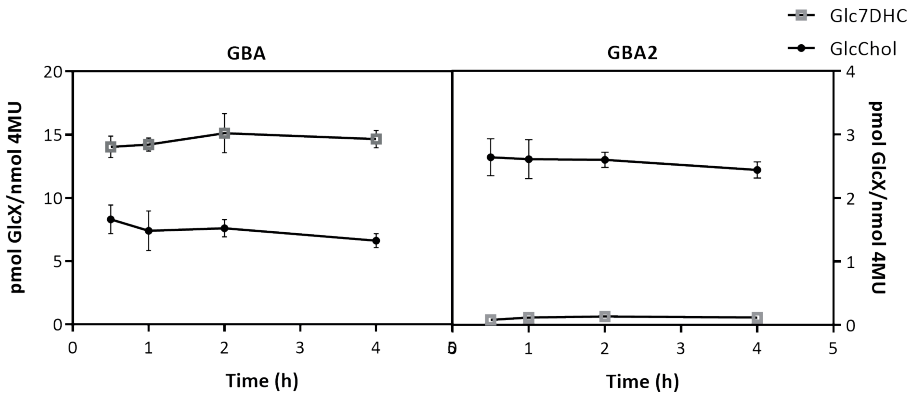


Figure 3. Ratio Transglucosylation and hydrolase activities. *rGBA was incubated for varied times with cholesterol (Chol) or 7-dehydrocholesterol (7DHC) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc). 4MU-emmitted fluorescence (nmol 4MU/mg protein) was measured a LS-55 Fluorescence spectrometer (PerkinElmer) using λ_{EX} 366 nm and λ_{EM} 445 nm. Formation of GlcChol and Glc7DHC was measured by LC-MS/MS (pmol/µg protein). The measured pmol/µg protein, product of transglucosylation, is divided by the amount of nmol 4MU/µg protein, product of hydrolysis, giving the ratio between transglucosylation and hydrolase activities (pmol GlcX/nmol 4MU).*

As both 7DHC and Chol are good acceptors for the transglucosylation reaction, we studied competition of both acceptors. Our results show that rGBA has no specific preference for one of the acceptors. This is observed with both 4MUGlc and GlcCer as sugar donor (Figure 4).

Next, we studied degradation of pure Glc7DHC by rGBA and GBA2 through monitoring its reduction with LC-MS/MS (Figure 5). We observed rapid time-dependent degradation upon incubation with rGBA at 37°C, a degradation which was not observed for GBA2. Incubating GBA with Glc7DHC (4 µM) in the presence of GlcChol and GlcCer only showed competition when the latter lipids were present at concentrations close to 1 mM.

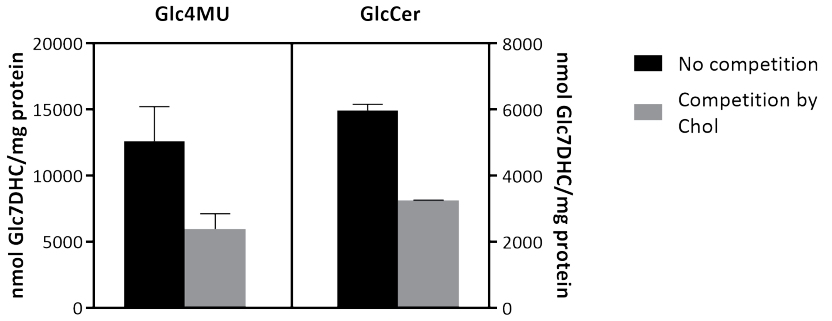


Figure 4. Competition of 7DHC and Chol for transglucosylation. *rGBA was incubated for 1 hour with 7DHC (0.3 mM) only or 7DHC (0.3 mM) and Chol (0.3 mM) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer). Formation of Glc7DHC was measured by LC-MS/MS (nmol/mg protein).*

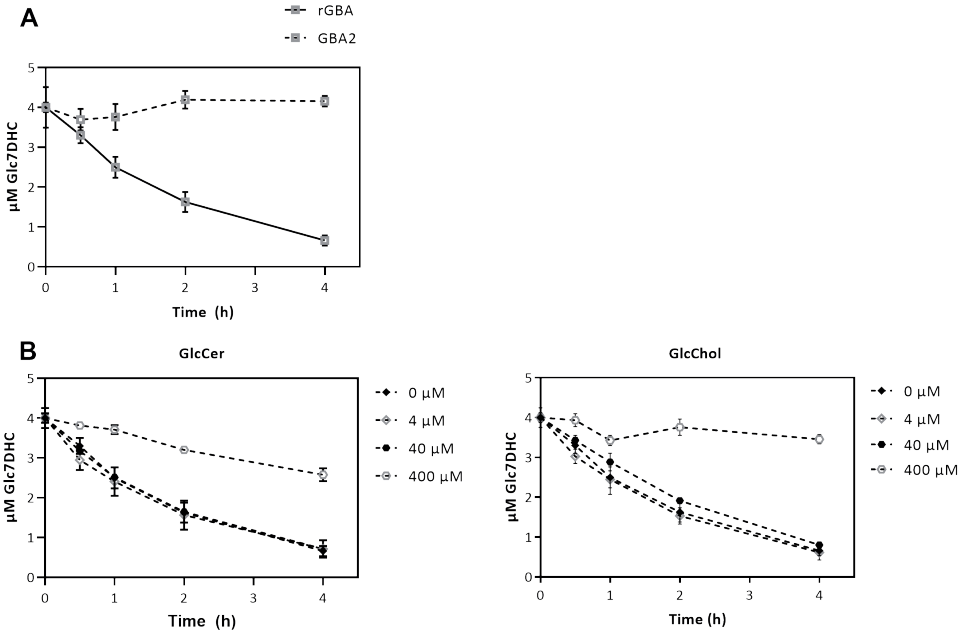


Figure 5. Degradation of Glc7DHC. *A) Either rGBA or GBA2 was incubated for varied times with 4 µM Glc7DHC. B) Influence of GlcCer or GlcChol, in concentrations of 4, 40 or 400 µM, on the degradation of Glc7DHC by rGBA are shown. Degradation of Glc7DHC was measured by LC-MS/MS (µM) and corrected for amount of protein.*

Conversion of Glc7DHC into $GlcD_3$

Within the skin 7DHC is converted to pre-cholecalciferol ($PreD_3$) and cholecalciferol (D_3) under influence of UVB irradiation (305 nm) and thermal dependent rearrangement of the double bonds in $PreD_3$ [7, 8]. It was studied whether glucosylated 7DHC can undergo the same conversion. For this, Glc7DHC was generated via a transglucosylation reaction between GlcCer and 7DHC, extracted by Bligh and Dyer and cleaned with butanol/water extraction. Glc7DHC was resuspended in methanol and irradiated with UVB for varied time at 37°C. Afterwards samples were measured on LC-MS/MS for detection of the compounds Glc7DHC (RT 1.29) and $GlcD_3$ (RT 1.19) (Figure 6). We detected that overtime Glc7DHC is converted into $GlcD_3$. Our data shows that next to the peak of Glc7DHC and $GlcD_3$ also another peak occurs in the chromatograms (Supplemental Figure 1). As the peak appears earlier in time than the peak for $GlcD_3$ and has the same mass as Glc7DHC and $GlcD_3$, we assume that this is $GlcPreD_3$ (Figure 6).

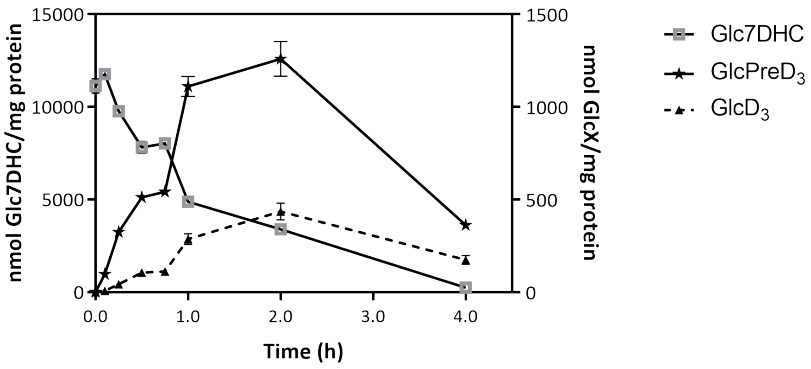


Figure 6. Conversion of Glc7DHC into $GlcPreD_3$ and $GlcD_3$. *Glc7DHC was irradiated with UVB for varied time. Samples were analysed by LC-MS/MS for the presence of Glc7DHC, $GlcD_3$ and the assumed $GlcPreD_3$.*

In vitro formation and degradation of $GlcD_3$ by transglucosylation by β-glucosidases

We studied whether D_3 itself is transglucosylated by GBA, GBA2 or GBA3. Recombinant GBA was incubated for 1 hour in 150 mM McIlvain buffer (pH 5.2), supplemented with Triton X-100 (0.1% v/v), sodium taurocholate (0.2% w/v), bovine serum albumin (0.1% w/v), 4MUGlc (3.7 mM) as glucose donor and D_3 (0.3 mM) as acceptor. Formation of glucosylated D_3 was monitored by LC-MS/MS. To determine optimal conditions, time and pH of incubation were varied. GBA was found to produce $GlcD_3$ over time (Figure 7A). We replaced 4MUGlc by GlcCer as potential glucose donor and observed similar formation of $GlcD_3$ (Figure 7A).

Optimal pH for the generation of the glucosylated metabolites was determined and found to be between pH 4.5 and pH 5.0 (Figure 7A), coinciding with the pH of lysosomes in which GBA normally resides.

Next, we incubated homogenates of cells overexpressing GBA2 with D_3 and 4MUGlc or GlcCer as glucose donors and analysed formation of $GlcD_3$. To exclude action of GBA, the homogenates were pre-treated with an irreversible GBA inhibitor, adamantyl-cyclophellitol. Optimal formation of $GlcD_3$ by GBA2 in the homogenates was low and observed at slightly higher pH as for rGBA (Figure 7B as compared to Figure 7A). Of note, when homogenates were incubated with D_3 without additional glucose donor (4MUGlc or GlcCer) hardly any $GlcD_3$ was detected. Apparently, GBA2 poorly generates $GlcD_3$, like Glc7DHC, at least as compared to GBA.

Finally, we investigated the broad-specific β-glucosidase GBA3. We did not observe detectable transglucosylation of $GlcD_3$ (data not shown).

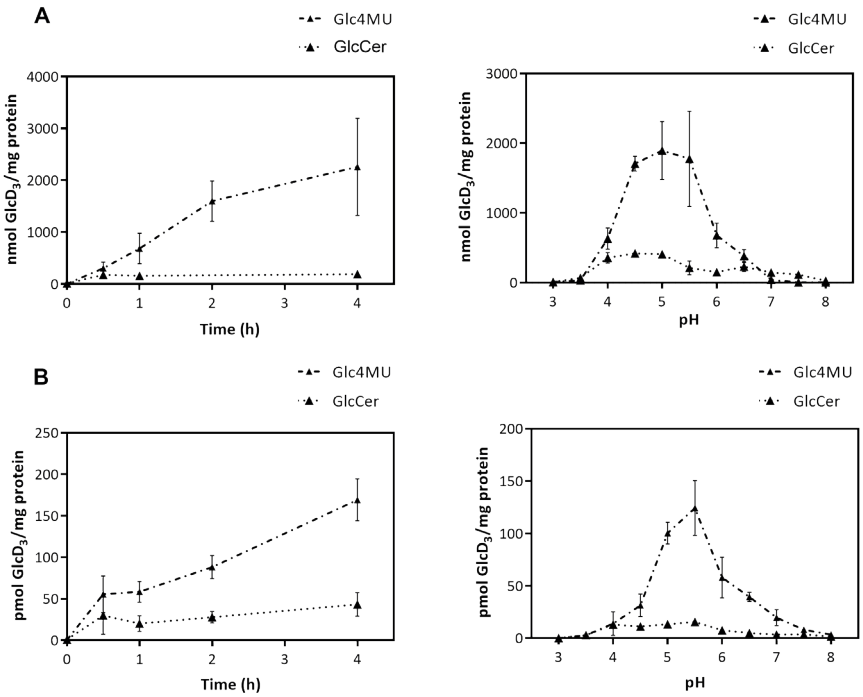


Figure 7. In vitro formation of $GlcD_3$. *A) rGBA was incubated for varied times and varied pH with cholecalciferol (D_3) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer) donor. Formation of $GlcD_3$ was measured by LC-MS/MS (nmol/mg protein). B) Lysates of cells overexpressing GBA2 were incubated for varied times and varied pH with D_3 in the presence of 4MUGlc or GlcCer donor. Formation of $GlcD_3$ was measured by LC-MS/MS (pmol/mg protein).*

For $GlcD_3$ the relative transglucosylation and hydrolase activities were also investigated. For this, experiments were conducted at optimal pH for GBA (pH 5.2) and separately for GBA2-enriched homogenate at optimal pH of 5.8. Besides the $GlcD_3$ levels also the formed 4-methylumbelliferone (4MU) was measured (Figure 8A). Both GBA and GBA2 seem to have difficulty with the transglucosylation reaction, as the ratio is strongly in favour of the hydrolysis of 4MUGlc.

We next studied whether GBA and GBA2 are able to degrade $GlcD_3$. Both GBA and GBA2 did not show ability to degrade $GlcD_3$ (Figure 8B).

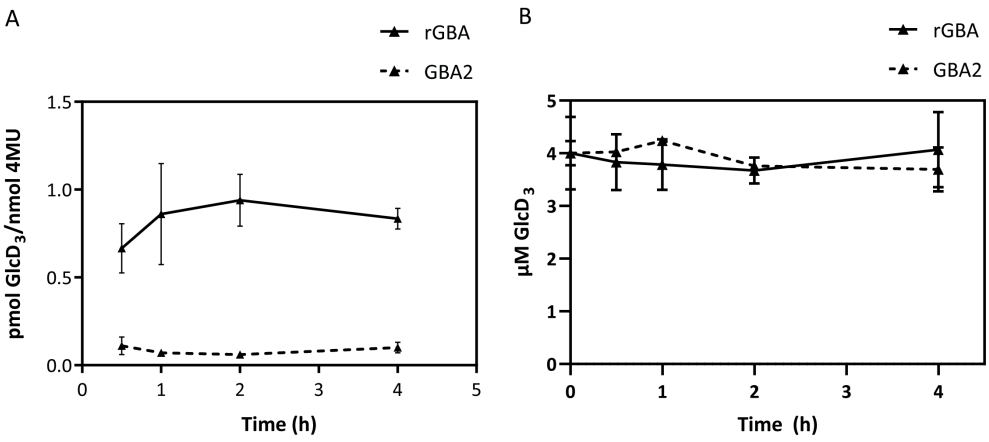


Figure 8. Ratio Transglucosylation and hydrolase activities and degradation of $GlcD_3$. *A) rGBA was incubated for varied times with cholecalciferol (D_3) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc). 4MU-emmitted fluorescence (nmol 4MU/mg protein) was measured a LS-55 Fluorescence spectrometer (PerkinElmer) using λ_{EX} 366 nm and λ_{EM} 445 nm. Formation of $GlcD_3$ was measured by LC-MS/MS (pmol/µg protein). The measured pmol/µg protein, product of transglucosylation, is divided by the amount of nmol 4MU/µg protein, product of hydrolysis, giving the ratio between transglucosylation and hydrolase activities (pmol $GlcD_3$/nmol 4MU). B) Either rGBA or GBA2 was incubated for varied times with $GlcD_3$. Degradation of $GlcD_3$ was measured by LC-MS/MS (µM) and corrected for amount of protein.*

Measurement of GlcChol, Glc7DHC and $GlcD_3$ in GD spleen

We investigated the presence of GlcChol, Glc7DHC and $GlcD_3$ in human spleen. For LC-MS/MS quantification internal standards $^{13}C_6$-GlcChol and $^{13}C_6$-Glc7DHC were used. Figure 9 shows GlcChol and Glc7DHC detection in human control spleen as well as in human GD spleen. $GlcD_3$ could not be detected. Levels of Glc7DHC are relatively low as compared to levels of GlcChol. In the spleens of type 1 GD patients elevated levels of GlcChol were observed, while levels of Glc7DHC were unchanged compared to the healthy spleens.

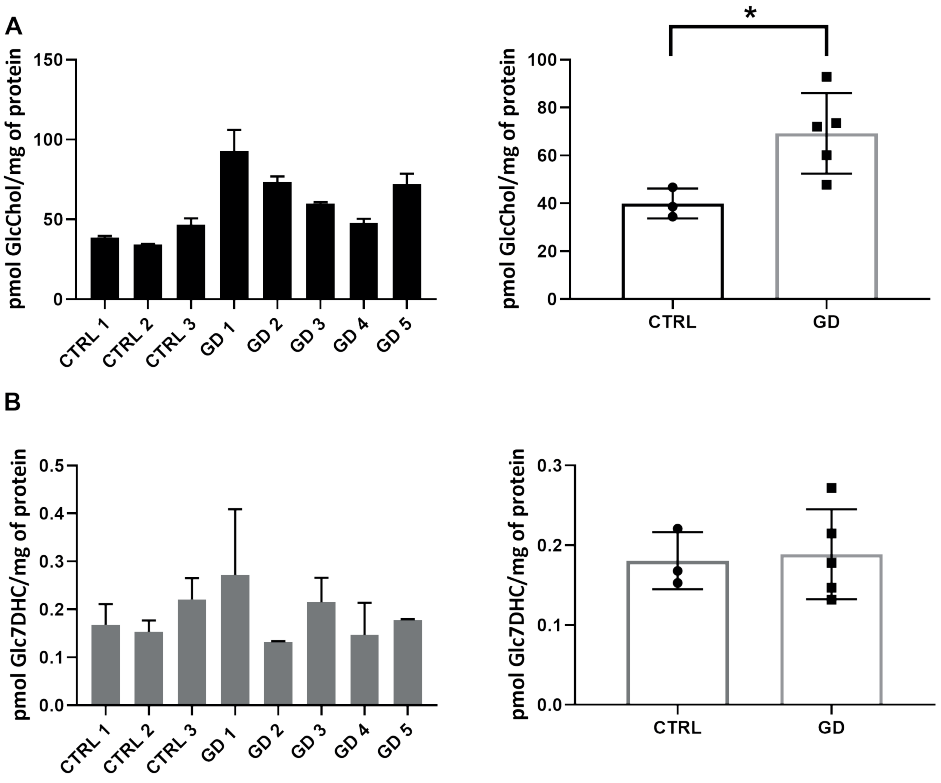


Figure 9. Levels of GlcChol and Glc7DHC in GD spleen. *LC-MS/MS analysis of GlcChol and Glc7DHC occurrence in human control spleens and human GD spleens. Errors bars are standard deviation of technical duplicates. Data were analysed by unpaired Student's t-test. P values <0.05 were considered significant (*P<0.05).*

Measurement of GlcChol, Glc7DHC and $GlcD_3$ in skin

As the pathway of D_3 occurs in skin, we investigated the presence of GlcChol, Glc7DHC and $GlcD_3$ within skin. The internal standards $^{13}C_6$-GlcChol and $^{13}C_6$-Glc7DHC were used for LC-MS/MS quantification. Table 1 shows GlcChol and Glc7DHC detection in skin of three healthy female individuals, ages ranging from 21 – 50 year, and skin colour ranges from white to dark. $GlcD_3$ was not detected. For GlcChol an age dependence can be observed, as patient number 1 (21 years of age) and patient 3 (23 years of age) show comparable levels of GlcChol. The age dependence was not observed for Glc7DHC. For Glc7DHC a relation with increasing skin pigmentation is observed, as increasing skin pigmentation shows an increase in Glc7DHC level. For confirmation of the data a lager cohort of samples is required.

Table 1. Detection of GlcChol and Glc7DHC in skin. *Age, skin colour, levels of GlcChol and Glc7DHC (pmol/mg tissue) per patient (female).*

Patient nr	Age	Skin colour	GlcChol pmol/mg tissue	Glc7DHC pmol/mg tissue
1	21	White	204,8	2,8
2	50	Tanned	338,1	4,0
3	23	Dark	265,7	6,0

Discussion

In previous work we developed a sensitive method to measure GlcChol in *in vitro* and *in vivo* samples. With this method we showed that GlcChol is naturally occurring in mammals [71]. With small adjustments to this method, we could set up a similar sensitive method for measuring Glc7DHC. Our findings show that 7DHC is an excellent acceptor for transglucosylation by GBA.

GBA is found to be able with 4MUGlc as sugar donor to form both Glc7DHC and GlcChol. GBA is also able to degrade Glc7DHC. GBA2 is much less active towards 7DHC compared to GBA1. This sharply contrast to the prominent transglucosylation of Chol by GBA2, on a par with GBA1. The observed difference in affinity of GBA2 for Chol and 7DHC is remarkable given the structural similarities between both sterols.

While GBA2 appears to mediate formation of GlcChol with GlcCer as natural sugar donor and GBA1 under normal conditions degrades it, for Glc7DHC GBA seems largely responsible for both degradation as well as formation. This difference is reflected by the clear accumulation of GlcChol in GD spleen and the normal levels of Glc7DHC in the same tissues.

Regarding synthesis and degradation of $GlcD_3$ similar observations were made as for Glc7DHC. *In vitro*, GBA is able to generate $GlcD_3$ by transglucosylation and it is also able to degrade it. In contrast, GBA2 only marginally produces $GlcD_3$ from D_3 and seems not able to degrade it. While the presence of Glc7DHC in the skin was detected, no $GlcD_3$ could be detected in this tissue. It will be interesting to investigate GD skin on abnormalities in Glc7DHC and $GlcD_3$. This is particularly relevant the commonly reported osteoporosis in GD patients [83, 88-91].

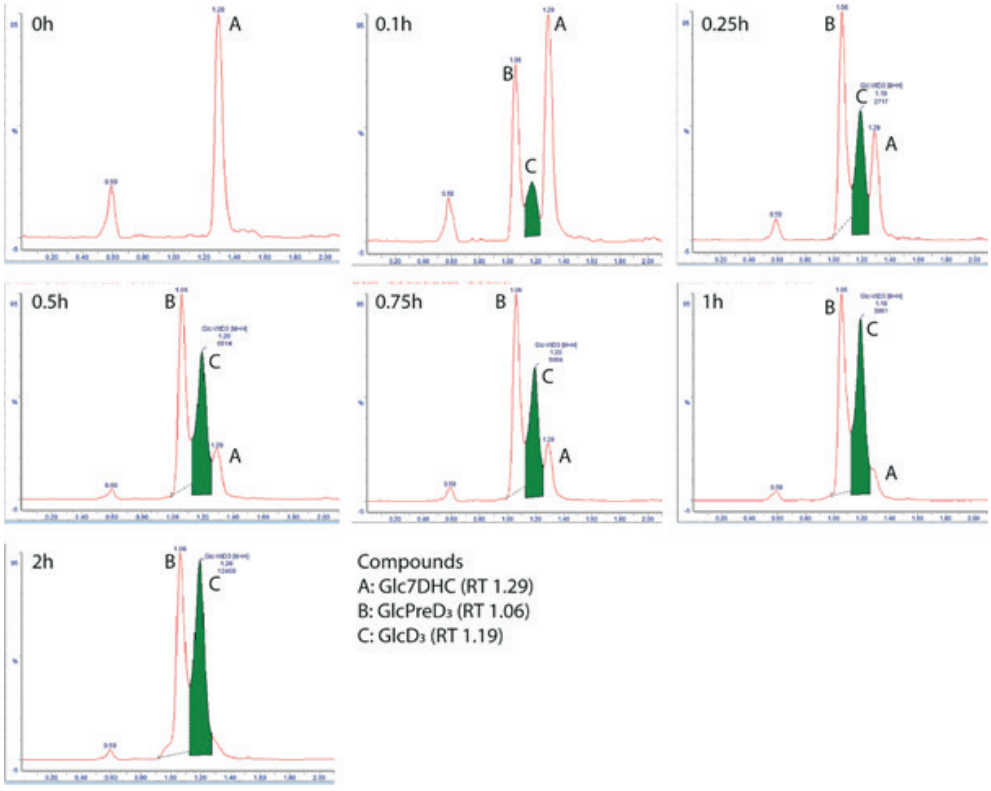
A final consideration is the partial overlap in symptoms between diseases such as SLOS, GD and NPC (Supplemental Table 1). It is appealing to comparatively document for these disorders the presence of glucosylated compounds such as GlcChol, Glc7DHC and $GlcD_3$. Osteoporosis and vitamin D_3 abnormalities are reported for GD that theoretically might be related to abnormal 7DHC and D_3 metabolism in the skin. We consider the possibility, that patients of SLOS, which have high levels of 7DHC are prone to develop high levels of Glc7DHC [64-68], on condition that excessive 7DHC is present in the lysosome. In GD and NPC this explanation is the foundation for the high levels of GlcChol that is detected in GD and NPC patients [71]. In all three diseases levels of GlcCer are elevated [71, 84, 85], presenting a good sugar donor for transglucosylation. The data on the GD spleens show that high levels of GlcChol are detected. As GBA is defect, it is expected that the excessive amount of GlcCer in the lysosomes is transported to outside of the lysosome, where GBA2 gets over stimulated to perform the transglycosylation reaction between the in lysosome present Chol and the excessive GlcCer, resulting in high levels of GlcChol. The data on the GD spleens

in relation to Glc7DHC, show that Glc7DHC levels are not influenced by GD. We expect that high levels of GlcCer outside the lysosome promote GBA2 to take over the role of the defect GBA to form Glc7DHC. Maintaining the levels of Glc7DHC on the same levels as in healthy people, by generating just enough as needed by the body, as Glc7DHC cannot be degraded by GBA2. As in SLOS patients no bone problems are observed, high levels of 7DHC are detected and high levels of Glc7DHC are expected, both 7DHC and Glc7DHC might be offering those patients protection.

Future work on levels of glucosylated compounds is needed to show the biological and pathological relevance of glucosylated compounds such as GlcChol, Glc7DHC and $GlcD_3$ in those diseases. Glucosylated metabolites might be more important for disease development than we suspect so far.

Supplementary information

Supplemental figure 1. Chromatograms UVB irradiation of Glc7DHC over time. *Conversion of Glc7DHC into GlcPreD$_3$ and GlcD$_3$. All are in [M+H]$^+$ transition, showing the formation without need of zooming in onto the peaks of GlcPreD$_3$ and GlcD$_3$. Same phenomenon was observed in [M+NH$_4$]$^+$ transition (data not shown).*



Supplemental Table 1. Overlap in disease symptoms for SLOS, GD and Niemann Pick Diseases.

		SLOS	Gaucher Disease				Niemann Pick Disease	
			Type I	Type II	Type III	Perinatal Lethal (collodion baby)	Type C1	Type C2
OMIM		#270400	#230800	#230900	#231000	#608013	#257220	#607625
Malacards ID		SMT004	GCH015	GCH016	GCH017	GCH018	NMN015	NMN014
Facial features	Anteverted nares	**X**				**X**		
	Broad/flat nasal bridge	**X**				**X**		
	Micrognathia	**X**				**X**		
	Microcephaly	**X**				**X**		
	Low-set ears	**X**				**X**		
Skeletal	Osteoporosis		**X**					
	Osteosclerosis		**X**		**X**			
	Bone pain/bone crisis		**X** **(crisis)**		**X**			
	Fractures (osteomalcia)		**X** **(pathologic)**		**X**			
Neurologic Central Nervous	Hypotonia	**X**					**X**	**X**
	Mental retardation	**X**						

Experimental procedures

Materials

Pure grade chemicals used were: 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) purchased from Glycosynth™ (Winwick Quay Warrington, Cheshire, England), sodium taurocholate from (EMD Millipore Corp, Billerica MA, USA), D-glucosyl-β-1,1'*N*-oleoyl-D-*erythro*-sphingosine (C18:1-GlcCer) Avanti Polar Lipids (Alabaster, AL, USA), cholesterol and 7-dehydrocholesterol from Sigma-Aldrich (St Louis, MO, USA). The GBA1 inhibitor ME656 was synthesized in the department of Bio-organic Synthesis (University of Leiden, The Netherlands) as described earlier [86]

The following enzymes were used in the investigation: recombinant human glucocerebrosidase (*Cerezyme*) obtained from Sanofi-Genzyme Corp, lysates of HEK293T-cells overexpressing GBA2 or GBA3.

Pure grade solvents used were for LC-MS/MS: ethanol purchased from Honeywell|Riedel-de Haën™ (Muskegon, USA), LC-MS-grade methanol, 2-propanol, water and HPLC-grade chloroform from Biosolve, LC-MS butanol from Merck KGaA (Darmstadt, Germany) and LC-MS quality ammonium formate from Sigma-Aldrich (St Louis, MO, USA).

Spleen. During therapeutic splenectomy or at autopsy, surgical specimens of human spleen were obtained. Phenotype of the subjects was established by clinical examination. The spleens were stored at -80°C. From the frozen materials homogenates were prepared in water.

Skin. During cosmetic surgery breast skin was obtained from a local hospital, with consent. Within 24 h after surgery subcutaneous fat was removed from the full thickness skin, with a surgical scalpel. The remaining skin were stored at -80°C. The unfrozen skin was extracted and measured for glucosylated metabolites of interest.

Specific lipid standards. $^{13}C_6$-β-GlcChol [71], GlcChol [71], $^{13}C_6$-β-Glc7DHC, Glc7DHC, $GlcD_3$, $^{13}C_6$-β-GlcDesm and GlcDesm were synthesized at the Bio-organic Synthesis department (Leiden University, The Netherlands). The full description of the syntheses will be described separately.

Methods

In vitro assay of hydrolase activity. Recombinant GBA1 was incubated with 3.7 mM 4MU-β-Glc in the presence of Triton X-100 (0.1% v/v) and sodium taurocholate (0.2% w/v) in McIlvaine buffer (0.1 M citric acid/ 0.2 M Na_2HPO_4), pH 5.2, supplemented with bovine serum albumin (0.1% w/v) [87]. GBA2-rich cell lysate was incubated with 3.7 mM 4MU-β-Glc in 150 mM McIlvaine buffer, pH 5.8 [88]. The reactions were stopped by addition of excess NaOH-glycine (pH 10.3) and 4MU-emmitted fluorescence was detected with a LS-55 Fluorescence spectrometer (PerkinElmer) using λ_{EX} 366 nm and λ_{EM} 445 nm.

In vitro assay of transglucosylation activity. Recombinant GBA and lysates of HEK293T cells overexpressing GBA2 were used to determine transglucosylation activity of the enzymes GBA and GBA2. The assays were performed as described earlier [71] with a few modifications. The samples for activity measurement contained 40 µL of recombinant GBA diluted 1:1000 in 25 mM KPI buffer (pH5.2) supplemented with Triton X-100 (0.1% v/v) or 40 µL of homogenate of GBA2 overexpressing cells, to which was added 10 µL of 50 nM ME656 (GBA1 specific inhibitor) or McIlvain buffer (citrate-phosphate buffer, pH 5.8). The glucose donor was varied in experiments with final concentrations in the reaction of 100 µM C18:1-GlcCer or 2.8 mM 4MUGlc. The acceptor was also varied: either a final concentration in the reaction of 0.3 mM Chol, 0.3 mM 7DHC or 0.3 mM D3 were used. The transglucosylation reaction in the case of recombinant GBA was carried out in the presence of additional 200 µL of a 150 mM McIlvain buffer (citrate-phosphate buffer) pH 5.2 containing 0.1% BSA (w/v), 0,1% Triton-X-100 (v/v) and 0.2% sodium taurocholic acid (w/v) and 12.5 µL of ethanol.

The same reaction in the case of GBA2-rich cell lysate was performed in the presence of additional 207.5 µL 150 mM McIlvain buffer pH 5.8 and 5 µL of ethanol. Transglucosylation reactions were carried out by 1h incubation at 37°C, after which the reaction was terminated by putting samples on ice. To measure in parallel hydrolase activity an aliquot of 5 µL of each sample was taken, 295 µL NaOH-glycine (pH 10.3) was added and fluorescence was determined as described above. The residual sample was subjected to lipid extraction according to Bligh and Dyer [89] by addition of methanol, chloroform and water (final volumes: 1:1:0.9, v/v/v). Internal standards were added as 50 µL aliquots of 0.1 pmol/µL $^{13}C_6$-β-GlcChol and/or $^{13}C_6$-β-Glc7DHC each in methanol. The lower lipid phase was taken to dryness under vacuum at 45°C in a *speed FAC concentrator plus.* The isolated lipids were purified by butanol/water extraction (1:1, v/v) and the upper phase was taken to dryness under vacuum at 45 °C in a *speed FAC concentrator plus*. The isolated lipids were resolved in methanol and analysed by LC-MS as described in **Chapter 2** of this thesis.

Degradation of Glc7DHC and $GlcD_3$. The ability of recombinant GBA and homogenates of HEK293T cells overexpressing GBA2 to degrade Glc7DHC and $GlcD_3$ was monitored over time. Therefore 4 µM of Glc7DHC or $GlcD_3$ was added to 40 µL recombinant GBA diluted 1:1000 in 25 mM KPI buffer (pH5.2) supplemented with Triton X-100 (0.1% v/v) or 40 µL of homogenate of GBA2 overexpressing cells, to which was added 10 µL of 50 nM ME656 (GBA1 specific inhibitor) or McIlvain buffer (citrate-phosphate buffer, pH 5.8). The reaction in the case of recombinant GBA was carried out in the presence of additional 200 µL of a 150 mM McIlvain buffer (citrate-phosphate buffer) pH 5.2 containing 0.1% BSA (w/v), 0,1% Triton-X-100 (v/v) and 0.2% sodium taurocholic acid (w/v) and 12.5 µL of ethanol. The same reaction in the case of GBA2-rich cell lysate was performed in the presence of additional 207.5 µL 150 mM McIlvain buffer pH 5.8 and 5 µL of ethanol. To monitor the effect of the presence of GlcChol, GlcCer and 4MUGlc on Glc7DHC levels, 4 µM of these compounds were individually combined with 4 µM of Glc7DHC. All samples were prepared for LC-MS/MS as described previously (**Chapter 2**) and measured by LC-MS/MS for Glc7DHC or $GlcD_3$ levels.

Conversion of Glc7DHC into $GlcD_3$. Glc7DHC was irradiated with UVB (UVB lamp, Ushio 3000318 G8T5E TL 8W UV-B (306 nm) 288 mm) to examine whether the conversion of Glc7DHC into $GlcPreD_3$ and $GlcD_3$ takes place. First Glc7DHC was generated by transglucosylation reaction with recombinant GBA as described above. After clean-up by Bligh and Dyer and butanol/water extraction the samples were resuspended in 150 µL of methanol and irradiated by UVB for varied time. Afterwards samples were analysed by LC-MS/MS for the presence of Glc7DHC (RT1.29) and $GlcD_3$ (RT1.19). The measured peak at RT1.06 is assumed to be $GlcPreD_3$.

Measurement and quantification of GlcChol, Glc7DHC and $GlcD_3$ in spleen. The glucosylated sterols were extracted from spleen in accordance with the method of Bligh and Dyer [89] with a few modifications. Homogenised spleen (50 µL) was pipetted in an Eppendorf tube (2 mL). As internal standards 25 µL of 0.1 pmol/µL $^{13}C_6$-β-GlcChol and 0.1 pmol/µL $^{13}C_6$-β-Glc7DHC each in methanol, were added. The procedure of Bligh and Dyer and butanol/water clean-up were continued as described in **Chapter 2**.

Measurement and quantification of GlcChol, Glc7DHC and $GlcD_3$ in human skin. In accordance with the method of Bligh and Dyer [89] with a few modifications the glucosylated sterols of interest were extracted from full thickness skin. Skin was cut in small pieces (100-150 mg) with a surgical scalpel within an Eppendorf tube (2 mL). Internal standards were added as 50 µL aliquots of 0.1 µM $^{13}C_6$-β-GlcChol and 0.1 µM $^{13}C_6$-β-Glc7DHC each in methanol. Accordingly, to the described procedure of Bligh and Dyer and butanol/water clean-up in **Chapter 2**, skin samples were prepared for LC-MS/MS measurement.

Protein determination. The Pierce BCA Protein Assay kit (Thermo Scientific) was used for measurement of protein. Absorbance at 562 nm was measured in EL808 Ultra Microplate Reader (BIO-TEK Instruments Inc.).

Statistical analysis. Values in figures are presented as a mean ± S.D. Data were analyzed by unpaired Student's t-test. P values <0.05 were considered significant (*P<0.05).

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