Discovery and characterization of new glucosylated metabolites: pathophysiological consequences

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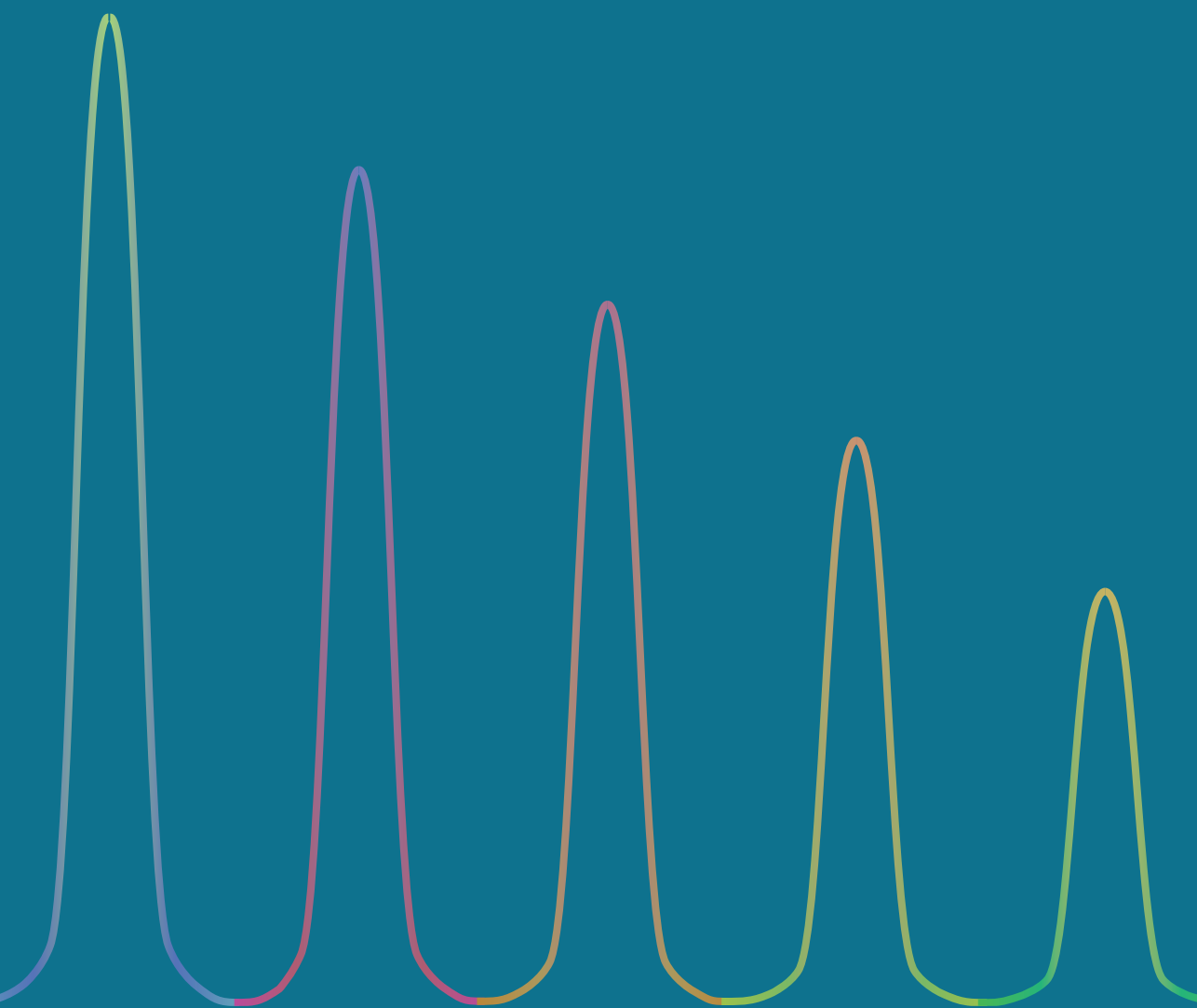
"Develop a passion for learning.
If you do, you will never cease to grow."

Anthony J. D'Angelo



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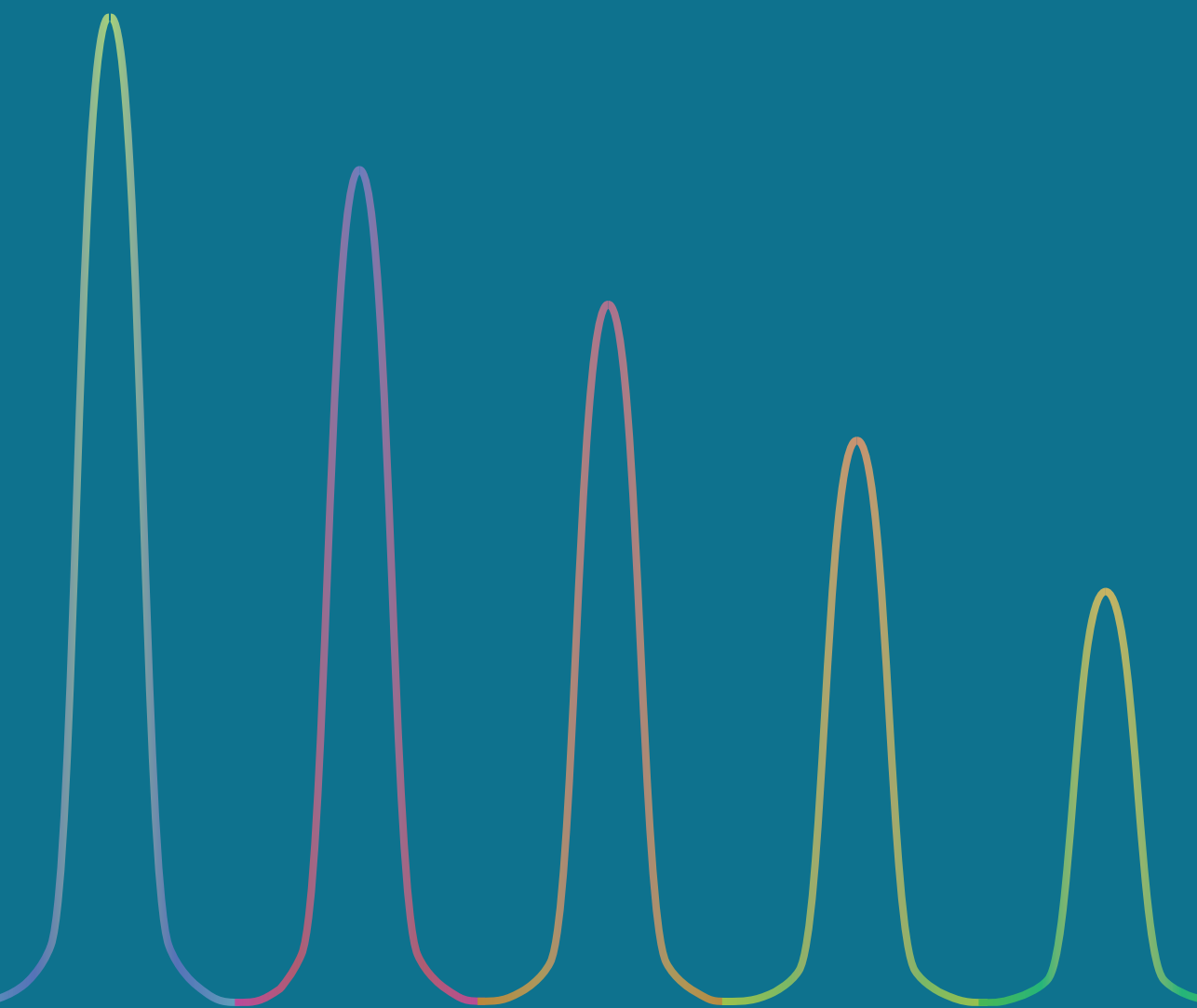


Chapter 1

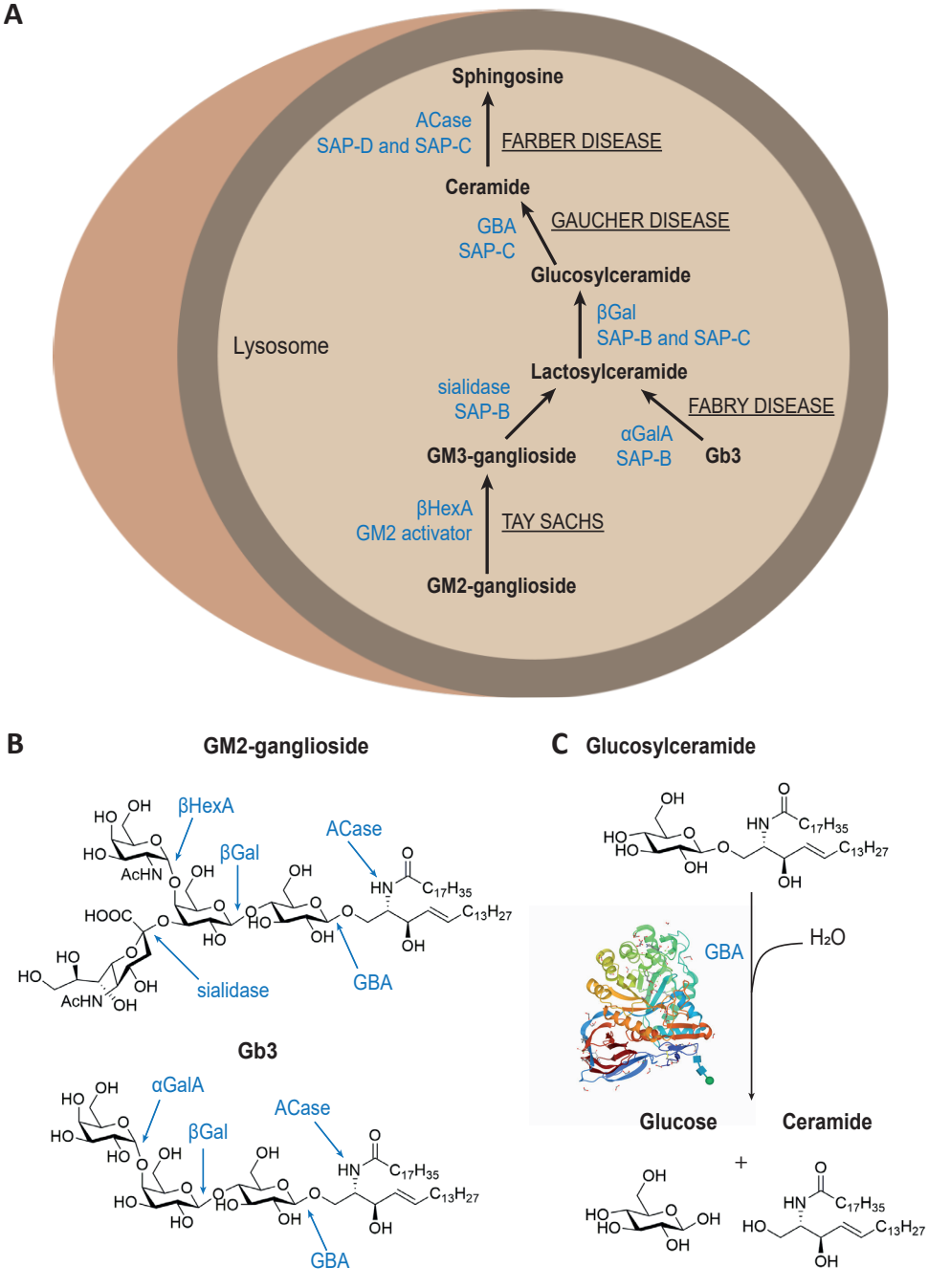
Introduction and outline

Lysosomes and lysosomal diseases

In the human body lysosomes, the perinuclear acid compartments of cells, are responsible for recycling macromolecules, amongst which complex glycoconjugates such as glycosphingolipids (GSLs) [1, 2]. Lysosomes are equipped for this latter degradation process with a broad cocktail of glycosidases (a.k.a. glycohydrolases) [3]. In 1884 the German chemist Johannes Ludwig Thudichum was the first to describe GSLs, a class of lipids with combined presence of fatty acid, amino acid and sugar elements. The prototype glycosphingolipid is glucosylceramide (GlcCer), a structure composed of variable glycan, fatty acid and sphingosine moieties. It acts as precursor for a large number of distinct GSLs [4, 5]. The lysosomal degradation of GLSs is a sequential cleavage process orchestrated by several glycosidases assisted by specific accessory proteins, named GM2 activator, Sphingolipid Activator Protein (SAP) B, C and D (Figure 1A). Sugar moieties are cleaved off one by one, eventually resulting in the ceramide lipid backbone (Figure 1B) [6, 7]. The responsible hydrolase, assisted by the accessory protein SAP-C, for removing the last glucose (Glc) from the ceramide (Cer) is the retaining β-glucosidase glucocerebrosidase (GBA) (Figure 1C). Inside the lysosome, GBA is assisted in activity towards GlcCer by the activator protein saposin C [6]. GBA is encoded by the *GBA* gene at locus q21 of chromosome 1 [8-10]. Mutations in this gene are associated with Gaucher Disease (GD), a relatively common lysosomal storage disorder (LSD). The French physician, Philippe C.E. Gaucher was the first to describe a GD patient. In his doctorate thesis he documented the symptoms of a young female with unexplained massive splenomegaly [11]. Soon after, it became that the patient represented a distinct disease entity that got named Gaucher's Disease. It took 80 years to identify that the molecular cause for the characteristic GlcCer accumulation in GD, being an inherited deficiency in the lysosomal acid β-glucosidase, later known as glucocerebrosidase [10, 12, 13]. There are over a 200 known mutations in the *GBA* gene linked to GD [14]. Yet, the clinical severity of the GD patients is poorly predicted by their genotype [15]. The presence of one allele encoding N370S GBA, common in Europe and among Ashkenazim, is always associated with a non-neuronopathic course of GD (the so-called type 1 variant). Otherwise, the correlation between GBA mutations and GD severity is limited. Besides the type 1 variant, there is an acute (infantile) and sub-acute (juvenile) neuronopathic form of Gaucher disease. The most extreme manifestation is the collodion baby with acutely lethal skim permeability abnormality incompatible with life beyond the womb. The latter severely affected patients always show very little residual GBA activity [10]. More recently it is recognized that mutations in the GBA gene, even at carrier level, are associated with an increased risk for Parkinson disease (PD) and Lewy-body dementia. Carriers of a mutant GBA allele show a 20-30 fold increased risk for developing PD [16]. The cause for this is still elusive. The common hepatosplenomegaly in GD patients is associated with local presence of Gaucher cells. However, many other symptoms, ranging from fatal skin defects to, bone disease or an almost asymptomatic course of disease are not clearly linked to GBA genotypes [10, 17].

Even GD patients with similar GBA genotypes, like monozygotic twins have, may differ in disease severity [18, 19]. Besides GD there are several other known LSDs with a genetic defect in hydrolases causing toxic lysosomal storage of specific glycosphingolipids, like Tay Sachs disease (TSD), Fabry disease (FD) and Farber disease (Figure 1D).

Patients with Tay Sachs disease (TSD) store the glycosphingolipid GM2-ganglioside due to a defect in β-hexosaminidase A (HexA) [20-22]. The disease is a progressive neurodegenerative disorder. Based on severity and age of disease onset, TSD has three different types [23]. The first, most studied and more often occurring form, is the (severe) infantile form. Characterized by onset up to 6 months after birth, and very low βHexA enzyme activity (<0.5%), resulting in death at the age of 2 or 3 years. The second form is the juvenile form with an age of onset of 3 – 10 years and most do not live past 15 years [24]. The third form is the less severe adult form, which shows a wider range of symptoms [25]. In both forms a partial deficiency of βHexA causes the disease to develop [24, 26-28]. Unlike GD, the broad disease heterogeneity in severity of symptoms and the age of onset is related to specific mutations influencing enzymatic activity of βHexA, as only 10-15% of βHexA activity is enough to prevent accumulation of GM2 ganglioside [27, 29].


A
Sphingosine
ACase
SAP-D and SAP-C
FARBER DISEASE
Ceramide
GBA
SAP-C
GAUCHER DISEASE
Glucosylceramide
βGal
SAP-B and SAP-C
Lysosome
Lactosylceramide
sialidase
SAP-B
FABRY DISEASE
αGalA
SAP-B
GM3-ganglioside
Gb3
βHexA
GM2 activator
TAY SACHS
GM2-ganglioside
B
GM2-ganglioside
βHexA
βGal
ACase
sialidase
GBA
Gb3
αGalA
βGal
ACase
GBA
C
Glucosylceramide
GBA
H₂O
Glucose
Ceramide
+

D

Disease	OMIM ID	Deficient Hydrolases	BRENDA ID	Primary Storage Products
TAY SACHS	OMIM #272800	βHexA	EC 3.2.1.52	GM2-ganglioside
FABRY DISEASE	OMIM #301500	αGalA	EC 3.2.1.22	Globotriaosylceramide + Globotriaosylsphingosine
GAUCHER DISEASE		GBA	EC 3.2.1.45	Glucosylceramide + Glucosylsphingosine
Adult type I	OMIM #230800			
Infantile type II	OMIM #230900			
Juvenile type III	OMIM #231000			
Collodion baby	OMIM #608013			
FARBER DISEASE	OMIM #228000	ACase	EC 3.5.1.23	Ceramide

Figure 1. Lysosomal degradation and related lysosomal storage diseases.
A) Schematic overview of lysosomal degradation of GM-2 and Gb3 glycosphingolipids. In blue the hydrolases: β-Hexosaminidase A (HexA), sialidase , α-galactosidase A (αGalA), GalCer β-galactosidase (βGal), glucocerebrosidase (GBA), lysosomal acid ceramidase (ACase) and the specific accessory proteins: GM2-activator protein and saposins B, C and D. B) Lysosomal degradation of ganglioside GM2 and globoside globotriaosylceramide (Gb3). C) Protein structure and degradation of glucosylceramide (GlcCer) into glucose and ceramide by GBA. D) Overview of lysosomal storage diseases and their defective hydrolase.

Fabry disease (FD) is a X-linked disorder that is characterized by intralysosomal accumulation of globotriaosylceramide (Gb3, ceramide trihexoside) [30, 31], due to inherited defects in lysosomal acid α-galactosidase (αGalA), which is encoded by the *GALA* gene [15, 31]. Like GD and TSD the clinical manifestations are very heterogeneous [32]. Males with classic FD develop at young age skin angiokeratoma, acroparesthesias (tingling, burning, numbness or stiffness or extreme episodic pains in the extremities) and anhidrosis (inability to sweat). Later in life this is followed by renal, cardiovascular and neurological impairment. Attenuated forms of the disease without the characteristic early disease signs and renal complications are observed in some female Fabry heterozygotes. Atypical variants of FD have been recognized recently. These individuals manifest single late onset organ complications in kidney or heart, due to mutations/ polymorphisms in the *GALA* gene [31, 33].

Farber disease, also known as lipogranulomatosis, is caused by a mutation in the *ASAH1* gene, resulting in a deficiency in the lysosomal acid ceramidase (ACase) [34-36]. Patients show distinct clinical symptoms due to the accumulation of ceramide in tissue [37, 38]. Clinical manifestations are subcutaneous skin nodules near the joints, resulting in pain and progressive joint stiffness, motion limitations by contractures and finally immobilizations and deformation of joints [37, 39]. Depending on the residual ACase activity patients show variety in central nervous system disease, leading to progressive neurologic deterioration, such as seizures, paralysis, myoclonus (involuntary muscle jerks) and loss of speech [38, 40, 41]. Farber disease is juvenile as the first symptoms usually appear before the first birthday. Milder forms have an onset of 20 months of age and neurologic deterioration has an onset of 1 to 2½ years of age. Patients die mainly within the first years of life [39, 42].

The development of treatments for LSDs has received considerable attention in recent decades. GD is the frontrunner in this field [15]. For non-neuronopathic type 1 GD patients allogeneic bone marrow transplantation (BMT) has proven to be a successful treatment. However due to limited availability of matching donors and the risks and invasive nature of transplantation this treatment is exploited minimally. A new and promising avenue is gene therapy based on genetically corrected autologous hematopoietic stem cells [43]. Meanwhile both enzyme replacement therapy (ERT) and substrate reduction therapy (SRT) have proven to be successful treatments for type 1 GD patients. For GD ERT use is made of macrophage-targeted recombinant human GBA containing glycans with terminal mannose moieties. This allows binding to the mannose-receptor expressed at the surface of tissue macrophages. By endocytotic uptake the enzyme is delivered to the lysosomes of macrophages. ERT reduces pathological Gaucher cells in peripheral tissues dramatically, reflected in major improvements in organomegaly and hematological abnormalities [15]. Drawbacks for ERT are the extreme costs and the need for individualized enzyme dosing regimens [44]. The alternative, SRT treatment, in which small compound inhibitors of glucosylceramide synthase (GCS), (Miglustat, Eliglustat) are orally administered, results in clinical responses [45-49]. The oral administration is seen as the biggest advantage of SRT over ERT [44].

If we compare the treatment of GD to the treatment of TSD, FD and Farber disease, GD has the best treatment options. In the case of FD, ERT treatment uses recombinant GLAs with mannose-6-phophate containing N-linked glycans that upon infusion are delivered to lysosomes of many cell types via mannose-6-phosphate receptor uptake [50, 51]. Unfortunately, the clinical effectiveness of FD ERT is far less than observed for type 1 GD ERT. Formation of neutralizing antibodies in enzyme-lacking FD males is the complicating factor for this treatment [52]. A recent new development is the first-in-class, small-molecule pharmacological chaperone Migalastat. An oral drug that binds to and stabilizes

amenable mutant forms of αGalA, to promote folding and transport from the endoplasmic reticulum to the lysosome. After dissociation from the transporting receptor, αGalA can degrade its substrate GBA3 in lysosomes. Switching from ERT to Migalastat has proven to be safe and therefore of great potential [53, 54]. TSD and Farber disease patients have the fewest treatment options. For TSD there is only treatment for late onset Tay Sachs, with low dose of pyrimethamine to increase βHexA activity. However, the treatment was only potential if given to patients with early stage TSD [27]. Current therapeutic approaches such as ERT, SRT, BMT, hematopoietic or neural stem cell transplantation and gene therapy have shown low efficacy to prevent the neurodegeneration or still need further investigation [27, 28]. Farber disease patients can only be treated when there is no neurological condition. In that case transplantation of hematopoietic stem cells are a source for reaching sufficient amounts of enzyme [39]. A proof of concept study in mice, has revealed a new potential treatment for selective acid sphingomyelinase inhibitors that have shown to ameliorate FD manifestations [55].

GBA: catalytic features

Since the first half of the 20th century glycosidases are being categorized into two groups, inverting glycosidases or retaining glycosidases. Each group has a distinct catalytic mechanism determining the stereochemistry outcome of the hydrolysis. Daniel E. Koshland Jr. was the first to describe these mechanisms [56]. GBA, as a retaining glycosidase, employs the Koshland double displacement mechanism in which the S_N2 mechanism occurs twice. As result a product with a net retention of stereochemistry is produced [57]. The catalytic pocket of GBA is about 5.5 Å apart and is equipped with the catalytic acid/base (E235) and the nucleophile (E340) to allow the hydrolysis of GlcCer [58-60].The catalytic acid/base allows the protonation of the glycosidic oxygen of glucosyl moiety. Simultaneously the catalytic nucleophile attacks the anomeric carbon. As result a transient oxocarbenium transition state is formed, followed by the formation of a covalent substrate-enzyme intermediate. The second half of the reaction follows the steps of the first half of the reaction in reversed order. In case of hydrolysis the catalytic acid/base deprotonates a water molecule, which displaces the nucleophile to form again a second transient oxocarbenium transition state. Next, (re-inverted) glucose and ceramide are released from the pocket (Figure 2A) [57, 60].

Because the reaction mechanism is well understood, several mechanistic inhibitors and chemical probes that react with the enzyme's active site catalytic amino acid have been developed. Two widely used inhibitors are the suicide inhibitor Conduritol B Epoxide (CBE) and the later discovered natural irreversible inhibitor cyclophellitol [61-63]. In a mechanism-based manner β-glucosidases are irreversibly inactivated by CBE [61, 62]. CBE was used to create a Gaucher-like mouse model and to identify the active sites of α- and β-glucosidases [62, 64, 65]. Cyclophellitol is now synthetically available [66, 67], and also reacts in a

mechanism-based manner and with higher potency with β-glucosidases [68, 69]. As the reaction mechanism depends on enzyme activity rather than affinity alone, cyclophellitol has been the scaffold for the first true activity-based probe (ABP) for lysosomal glucosidase [59]. A BODIPY-substituted cyclophellitol is generated by a Cu(I)-catalyzed "click" reaction with a BODIPY-alkyne [59]. Later, other cyclophellitol and cyclophellitol aziridine-based ABPs containing fluorophores and/or biotin were developed [70]. ABP's are used for visualization of endogenous GBA by SDS-PAGE, fluorescence microscopy, fluorescence-activated cell sorting (FACS), inhibitor screening and diagnosis of GD [59]. Furthermore the cyclophellitol based inhibitors have proven to be useful for developing a neuropathic Gaucher model in zebra fish [71] and were used for *in vitro* experiments in this thesis. Adamantyl-cyclophellitol selectively inhibits GBA1 in the nanomolar range (apparent IC_{50} values = 1.0 nM) [71] , while AMP-DNM iminosugar is a selective GBA2 inhibitor [59, 72] (Figure 2B).

Already in 1994 a potential second function of GBA was noted, transglycosylation [73]. Vanderjagt *et al.* showed the ability of GBA to transfer the glucose moiety from 4-methylumbelliferyl-β-glucoside to retinol and other alcohols. But it was not until 2013 that more on transglucosylation was reported [74]. Akiyama *et al.* showed that GBA can transfer the glucose moiety from the sugar donor glucosylceramide (GlcCer) to the sugar acceptor cholesterol (Chol) (Figure 2A). The product of the transglucosylation is 1-O-cholesteryl-β-D-glucopyranoside (GlcChol) (Figure 2C) [74, 75]. Of note, GBA can not only synthesize GlcChol, but it is also able to degrade it. In parallel and independently, Marques and co-workers made similar observations for GBA and other cellular β-glucosidases that led to a later publication [75].

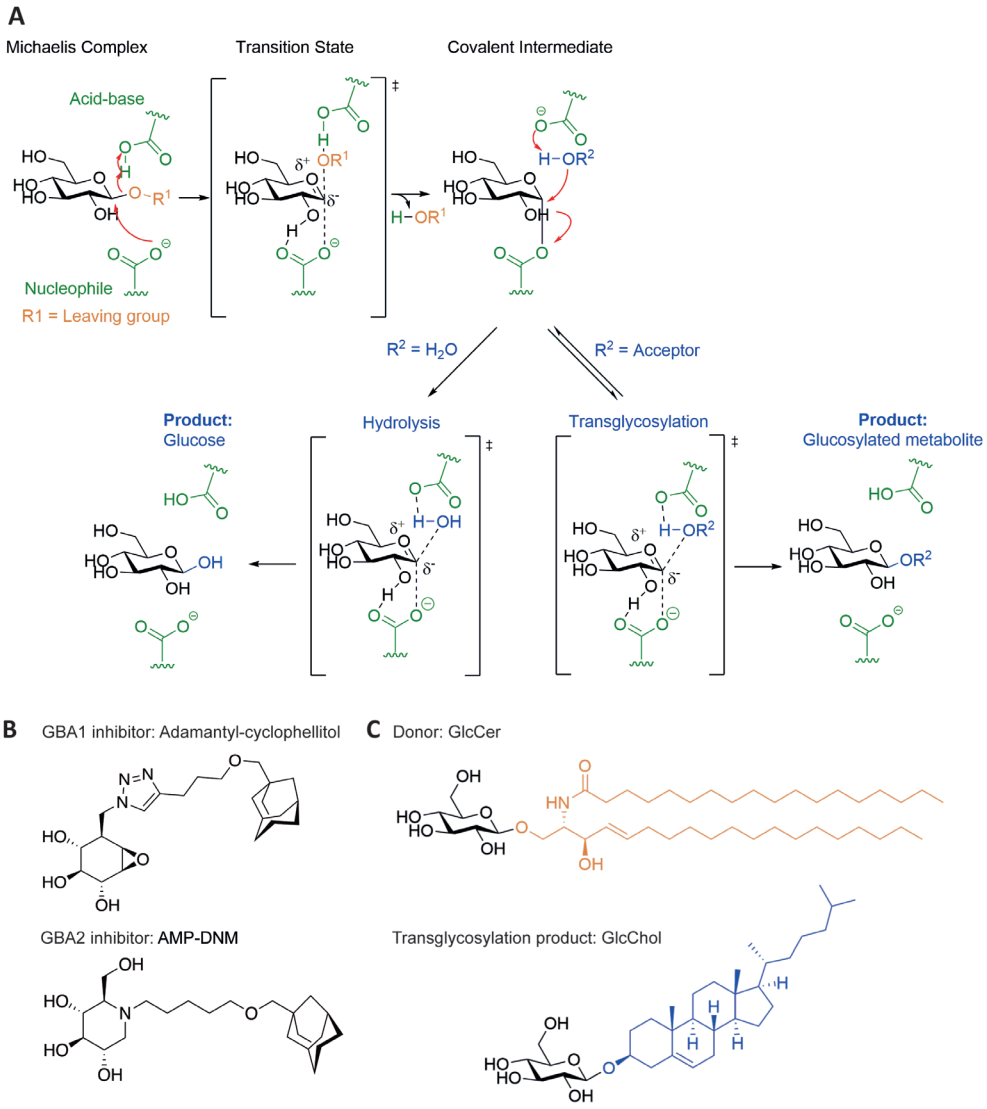


Figure 2. Reaction mechanism, inhibitors and transglycosylation.

A) Double displacement mechanism showing catalytic activity of GBA: hydrolysis and transglycosylation. ‡, transition state. R, aglycon. B) Structure of GBA1 inhibitor, adamantly-cyclophellitol and structure of GBA2 inhibitor, AMP-DNM. C) Structure of sugar donor glucosylceramide (GlcCer) for transglycosylation and 1-O-cholesteryl-β-D-glucopyranoside (GlcChol), the transglycosylation product.

GBA2 and GBA3

Besides GBA other β-glucosidases, that degrade GlcCer, are present in mammalian cells and tissues. Van Weely and co-workers firstly observed the existence of a second glucosylceramidase, being relative incentive to inactivation by CBE and not deficient in GD patients [76]. This tightly membrane-associated nonlysosomal glucosylceramidase (GBA2) is expressed in all cells [76-78]. GBA2 differs from GBA by being located outside lysosomes. It has been noted at the endoplasmic reticulum, Golgi apparatus and at the endosomes [77-79]. Another difference is the need for an activator protein. While GBA needs saposin C (SAP-C) for the degradation of GlcCer, GBA2 does not. Other differences between GBA and GBA2 are found in substrate specificity and inhibitor sensitivity [76]. Furthermore, GBA2 is not deficient in GD patients and there are indications for compensatory overexpression of activity [80]. In some tissues a third β-glucosidase, a broad-specific cytosolic β-glucosidase (GBA3), is expressed [81]. GBA3 has shown *in vitro* a relatively poor hydrolytic activity towards GlcCer, but is most likely involved in detoxification of glucosylated xenobiotics [81]. There is a common inherited deficiency in GBA3, but this seems not to be influencing severity of disease manifestation, at least as observed for type 1 GD patients [81].

Considering the formation of GlcChol by transglycosylation reaction and its degradation, GBA2 has shown to generate and degrade GlcChol. The data of Marques *et al*. implies GBA2 has a preference for transglycosylation, as levels of GlcChol are remarkably lower in tissues from mice with a GBA2-deficiency, while levels increase in tissues from mice with a GBA-deficiency. For GBA3 both reactions have not been observed [75]. Under normal conditions, GBA2 appears to primary synthesize GlcChol and GBA to primarily degrade it in lysosomes [72]. In the outer skin, the stratum corneum, extracellular GBA does perform the synthetic reaction and generates GlcChol [82]. In Niemann Pick disease type C (NPC), a disorder characterized by lysosomal cholesterol accumulation, the shear excessive concentration of cholesterol forces GBA to perform transglucosylation and generate GlcChol. Consequently, GlcChol is elevated in NPC tissues and plasma [75].

Physiological relevance of transglycosylation

First reports of natural occurrence of GlcChol were from fungi, bacteria and plants [83, 84] . The natural occurrence of GlcChol in mammalian cells was first observed in cultured human fibroblasts and gastric mucosa [85, 86]. Later it was also observed in mice and human tissues [75, 87, 88]. The physiological relevance of GlcChol is unclear. Of interest, it was observed that exposure of fibroblast to 42°C for 15 and 30 minutes leads to an increase in levels of GlcChol. The findings suggest a role for GlcChol in heat shock responses, and therefore assist cell survival [85]. Furthermore, significant levels of GlcChol (6 pmol/mg weight) are detected in the outer layer of the skin, the stratum corneum. The GlcChol detected in skin is likely metabolized by the present GBA, which transglucosylates the abundant GlcCer into cholesterol yielding GlcChol. The physiological function of GlcChol in the skin remains to be elucidated [82]. Within mice, levels of GlcChol could be related to disease. Elevated levels of GlcChol were detected in tissues of mouse models for GD and Niemann-Pick type C disease (NPC) [75]. Further research showed that elevated levels of GlcChol were present in patients of type 1 GD and NPC compared to healthy human individuals [75]. It was even shown that treatment of GD type 1 patients with SRT (Miglustat (a potent inhibitor of GBA2 as well) and Eliglustat) lowers such levels of GlcChol in plasma. Interestingly, patients treated with ERT (rGBA Cerezyme) did not reach the same extent of GlcChol reduction in plasma, although impressive clinical improvements were noted [75]. These findings support the concept that GCS-GBA2 are involved in synthesis of GlcChol whist normally GBA degrades it.

Acceptors in transglucosylation

The observed formation of GlcChol via transglucosylation raises the intriguing question whether other sterols besides cholesterol might comparably act as acceptors in transglucosylation. Key candidates in this respect are compounds similar in structure to cholesterol (Chol). Two metabolites which by virtue seem good candidates are the two direct precursors of Chol, desmosterol (Desm) and 7-dehydrocholesterol (7DHC).

Naturally sterols as Chol, Desm and 7DHC occur in our food, like vegetables, meat and dairy products [89-96]. Via the intestine the sterols are absorbed into the bloodstream and taken up by the liver [94, 95, 97, 98]. Together with de novo cholesterol synthesis dietary cholesterol absorption determines the balance for cholesterol homeostasis [98]. Cholesterol can be de novo synthesized from the very simple building block acetyl-CoA. The cellular cholesterol synthesis involves a complex chain of reactions catalyzed by more than 30 enzymes. Briefly, acetyl-coA is converted via isoprenoids to cyclical precursors of cholesterol [94, 99]. 3-Hydroxyl-3-methylglutaryl coenzyme A (HMGCoA), is a rate limiting enzyme in cholesterol biosynthesis that is the target of cholesterol-lowering statins. Another rate limiting enzyme is squalene mono-oxygenase, which forms the first cyclical intermediate in the pathway, lanosterol [100]. The major pathway

for cholesterol synthesis involves the Kandutsch-Russel pathway [101] that is initiated by the formation of lathosterol from lanosterol (Figure 3). The final two steps in this pathway are the conversion of lathosterol into 7DHC by the enzyme Sterol C5-desaturase (SC5D) and the conversion of 7DHC into cholesterol by 7-dehydrocholesterol reductase (DHCR7) [101-103]. The alternative pathway for the formation of Chol is the Bloch pathway where lanosterol is converted into subsequently dehydrolathosterol, 7-dehydrodesmosterol, and Desm (Figure 3). From Desm, Chol is generated by the enzyme 24-dehydrocholesterol reductase (DHCR24) [104]. The enzyme DHCR24 also impacts directly and indirectly on 7DHC. Firstly, it mediates transformation of 7-dehydrodesmosterol into 7DHC. Secondly, DHCR24 can stimulate DHCR7 activity which is responsible of a reduction in 7DHC levels: overexpression of DHCR24 increases DHCR7 activity, while knock down decreases DHCR7 activity [105].

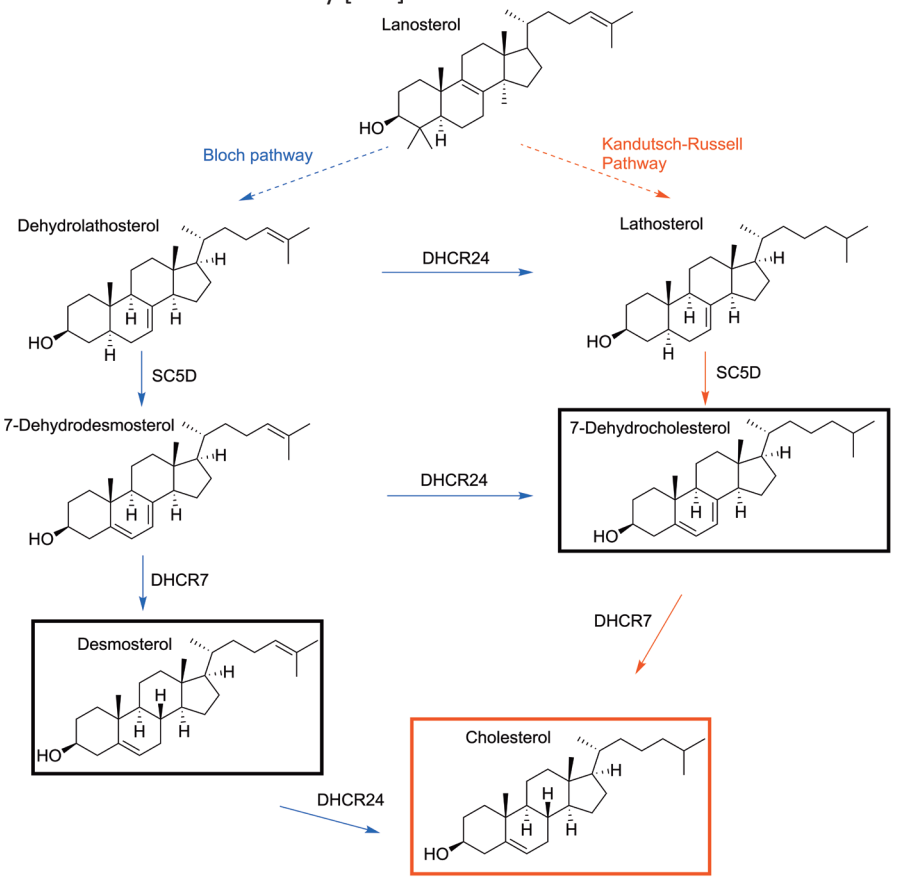


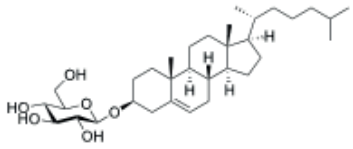
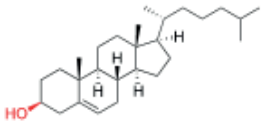
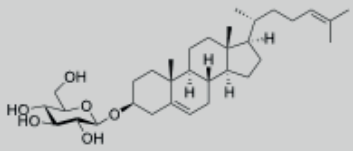
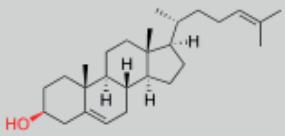
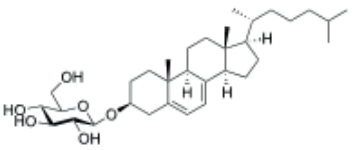
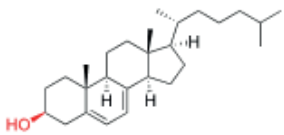
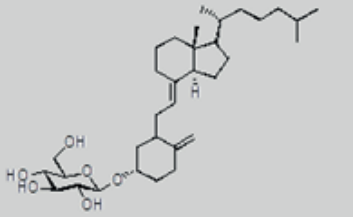
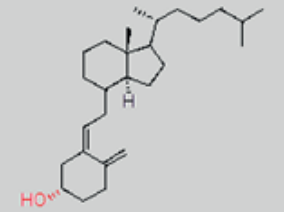
Figure 3. Cholesterol pathways.

Displaying both the Kandutsch-Russell Pathway and the Bloch pathway. Involved enzymes are sterol C5-desaturase (SC5D), 7-dehydrocholesterol reductase (DHCR7) and 24-dehydrocholesterol reductase (DHCR24).

All three structures of Chol, Desm and 7DHC are hydrophobic and show a secondary hydroxyl-group attached to the sterol scaffold (Table 1). This is a structure that is also observed in vitamin D (cholecalciferol, D_3), the downstream metabolite of 7DHC. In skin 7DHC is converted into pre-D_3 under influence of UVB irradiation (305 nm). A thermal dependent rearrangement of the double bonds of pre-D_3 results in the formation of D_3 [106, 107]. In this thesis we will investigate and report on the transglycosylation of new metabolites, such as Desm, 7DHC and D_3.

Table 1. Transglycosylation products and corresponding glucose donor and acceptor.

Presented are transglycosylation products: 1-O-cholesteryl-β-D-glucopyranoside (GlcChol), Glucosyl-desmosterol (GlcDesm), Glucosyl-7-dehydrocholesterol (Glc7DHC), Glucosyl-vitamin D (GlcD_3). Sugar acceptors: cholesterol (Chol), desmosterol (Desm), 7-dehydrocholesterol (7DHC), vitamin D (cholecalciferol, D_3). Sugar donor: glucosylceramide (GlcCer). Reference of literature in which glycosylated product has been reported.

Transglycosylation product	Acceptor	Donor	Literature Reference
GlcChol 	Chol 	GlcCer	[74, 75]
GlcDesm 	Desm 	GlcCer	
Glc7DHC 	7DHC 	GlcCer	
GlcD_3 	D_3 	GlcCer	

Goals of the thesis

The primary goal of this thesis is to investigate transglycosylation of cholesterol analogues. First the methodology to identify and detect the presence of glycosylated metabolites in natural materials is described. Secondly the formation and occurrence of glucosyl-desmosterol will be reported. Subsequently the investigation on the formation and occurrence of glucosyl-7-dehydrocholesterol and glucosylated vitamin D_3 is described. Furthermore, the role of GBA3 in formation and degradation of glycosylated metabolites is investigated.

Chapter 2. Reports on targeted discovery of glycosylated metabolites. Hereby ^{13}C-labeled were used in combination with LC-MS/MS methods to identify non-^{13}C-labeled glycosylated metabolites. The chapter describes the development of a (sensitive) LC-MS/MS method to quantify the glucosylated cholesterol (GlcChol) structural analogues named glucosylated desmosterol (GlcDesm), glucosylated-7-dehydrocholesterol (Glc7DHC) and glucosylated vitamin D_3 ($GlcD_3$). The established LC-MS/MS method allows the detection of these specific glucosylated metabolites in *in vitro* samples and biological materials. Allowing the further research on the biological presence and relevance of the GlcDesm, Glc7DHC and $GlcD_3$.

Chapter 3. Describes the formation and occurrence of glucosyl-desmosterol. The specific LC-MS/MS method allowed the demonstration of *in vitro* formation and degradation of GlcDesm by GBA and GBA2. Importantly, we detected GlcDesm within human spleen and were able to show that within spleen of Gaucher Disease patients elevated levels of GlcDesm are present. The research stimulates further research on glucosylated compounds within diseases, such as desmosterolosis and SLOS, which manifest symptoms that are reminiscent in GD.

Chapter 4. Describes the formation and occurrence of glucosyl-7-dehydrocholesterol and glucosylated vitamin D_3. The *in vitro* data shows that the specific LS-MS/MS method allowed demonstration of formation and degradation of both Glc7DHC and $GlcD_3$. Furthermore, the data shows the conversion of Glc7DHC into $GlcD_3$ by UVB-irradiation. Within biological samples, spleen and skin, Glc7DHC was detected. $GlcD_3$ on the other hand, could not be detected.

Chapter 5. Discusses the potential role of GBA3 in formation and degradation of glycosylated metabolites. No *in vitro* transglucosylation activity was observed for GlcChol. Furthermore, no degradation was observed for GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm.

Chapter 6. Discusses the obtained results of the performed research and describes future prospects of research, including the potential formation of xylosylated-7-dehydrocholesterol. Furthermore, the possibility of untargeted discovery of glycosylated metabolites is considered. Here for a so-called transbody was synthesized and tested for its potential to be transglycosylated by GBA and GBA2.

Other published contributions to the research field.

Important contributions regarding knowledge on glucosylated cholesterol were made in two studies performed at the department Medical Biochemistry at Leiden University that meanwhile are published. The papers are added as appendices:

Addendum I Marques AR, Mirzaian M, Akiyama H, Wisse P, Ferraz MJ, Gaspar P, Ghauharali-van der Vlugt K, **Meijer R**, Giraldo P, Alfonso P, Irún P, Dahl M, Karlsson S, Pavlova EV, Cox TM, Scheij S, Verhoek M, Ottenhoff R, van Roomen CP, Pannu NS, van Eijk M, Dekker N, Boot RG, Overkleeft HS, Blommaart E, Hirabayashi Y, Aerts JM. *Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular β-glucosidases.* J Lipid Res. 2016 Mar;57(3):451-63.

The paper reports the first demonstration of the occurrence of glucosylated cholesterol in human tissues. It reports the generation of GlcChol via transglycosylation by the enzyme GBA2. In addition, lysosomal GBA1 is shown able to similarly generate GlcChol in the presence of a large amount of cholesterol as acceptor (*in vitro* and in cells with high lysosomal cholesterol concentration). The own contribution to the work was focused on *in vitro* assays with recombinant human GBA1 assessing its hydrolase and transglucosidase activity.

Addendum II Boer DE, Mirzaian M, Ferraz MJ, Zwiers KC, Baks MV, Hazeu MD, Ottenhoff R, Marques ARA, **Meijer R**, Roos JCP, Cox TM, Boot RG, Pannu N, Overkleeft HS, Artola M, Aerts JM. *Human glucocerebrosidase mediates formation of xylosyl-cholesterol by β-xylosidase and transxylosidase reactions.* J Lipid Res. 2021;62:100018.

The paper reports on the existence of xylosylated lipids: xylosylated ceramide and xylosylated cholesterols. It is demonstrated that the enzyme glucosylceramide can form xylosylated ceramide from UDP-xylose and ceramide. Next, XylCer was found to be used by GBA1 to generate xylosylated cholesterol via transxylosylation. XylChol is relatively poorly hydrolysed by GBA1. Di-xylosylcholesterol generation by recombinant GBA1 could be detected. The occurrence of xylosylated lipids points to further catalytic versatility of GBA1. The own contribution was focused on *in vitro* assays with recombinant human GBA1 assessing its hydrolase and transxylosidase activity.

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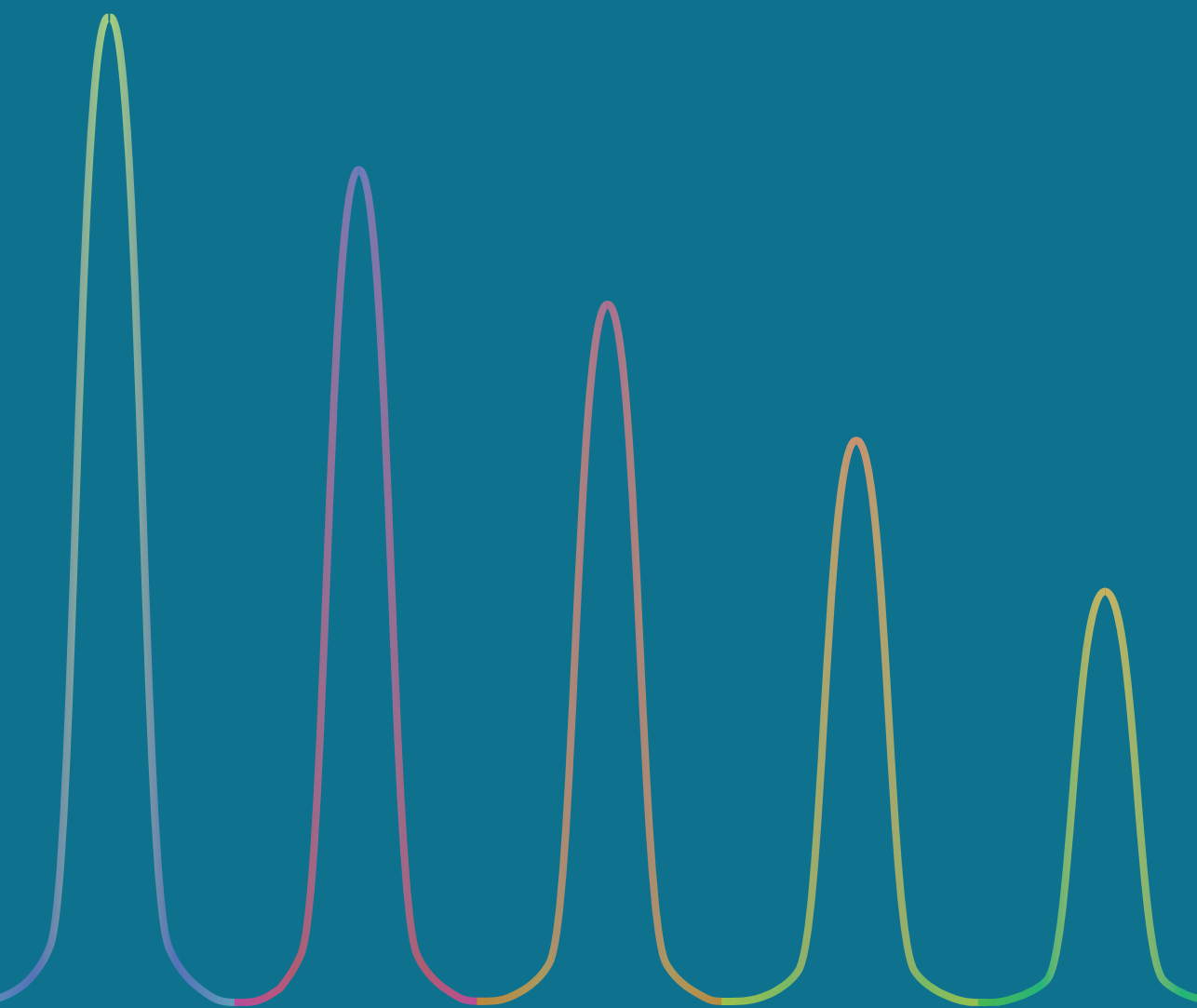


Chapter 2

Mass spectrometric quantification of glycosylated metabolites of cholesterol analogues using (isotope) standards

To be incorporated in revised form in an invited review

Chapter 2 - Mass spectrometric quantification of glycosylated metabolites of cholesterol analogues using (isotope) standards

Rianne Meijer, Patrick Wisse, Ken Kok, Mina Mirzaian, Maria J. Ferraz, Daphne E.C. Boer, Charlotte M. Beddoes, Marta Artola, Johannes M. Aerts.

To be incorporated in revised form in 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.A. : co-supervision
J.M.A. : supervision

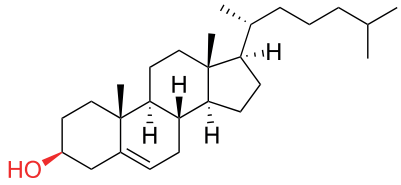
Abstract

Cholesterol (Chol) is an essential structural membrane lipid and an important precursor for metabolites such as oxysterols, bile acids and steroid hormones. The two analogues that resemble cholesterol very closely are its two precursors 7-dehydrocholesterol (7DHC) and desmosterol (Desm). The retaining β-glucosidase glucocerebrosidase (GBA) is able to transglucosylate the glucose moiety from glucosylceramide, the natural substrate of the enzyme, onto cholesterol, thus producing glucosylated cholesterol (GlcChol). A similar reaction is expected for its structural analogues, allowing the formation of glucosylated-7-dehydrocholesterol (Glc7DHC) and glucosylated desmosterol (GlcDesm). Expected is that Glc7DHC can be converted into glucosylated vitamin D (glucosylated cholecalciferol, $GlcD_3$) under influence of UVB irradiation (305 nm). To study these tentative glucosylated sterols, sensitive quantitative methods are required. We here report the development of a (sensitive) LC-MS/MS method to quantify Glc7DHC, $GlcD_3$ and GlcDesm in tissues and body fluids.

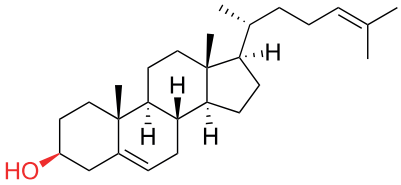
Introduction

The membrane-associated retaining β-glucosidase glucocerebrosidase (GBA, EC 3.2.1.45) is known for its deficiency in the lysosomal storage disorder Gaucher disease (GD) [1]. Defects in GBA impair its ability to degrade glucosylceramide (GlcCer) into glucose (Glc) and ceramide (Cer), resulting in lysosomal storage of GlcCer [2, 3]. Besides its function as a hydrolase, GBA has been shown to also catalyze transglycosylation [4-6]. This ability allows the formation of retinyl-β-D-glucoside (GlcRet) [4] and 1-*O*-cholesteryl-β-D-glucopyranoside (GlcChol) [5, 6]. Cholesterol (Chol) possess various structural analogues, with the sterols 7-dehydrocholesterol (7DHC) and desmosterol (Desm) showing the closest resemblance, both being the direct precursors of cholesterol in the cholesterol biosynthesis [7-9]. The existence of these closely related molecules, invites the investigation of the potential formation, and natural occurrence of glycosylated desmosterol (GlcDesm) and glucosylated 7DHC (Glc7DHC). As 7DHC is converted into vitamin D (cholecalciferol, D_3) under influence of thermal dependent rearrangement of the double bonds and UVB irradiation (305 nm) [10, 11], and as both 7DHC and D_3 have a secondary hydroxyl-group attached to their sterol scaffold, it makes D_3 an additional candidate for potential transglucosylation as well. Here we present LC-MS/MS methods for quantification of Glc7DHC, $GlcD_3$ and GlcDesm based on ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) using $^{13}C_6$-Glc7DHC and $^{13}C_6$-GlcDesm as the internal standards, which were synthesized by Dr. Patrick Wisse and Ken Kok (from the Bio-organic Chemistry Department, LIC) for this particular purpose.

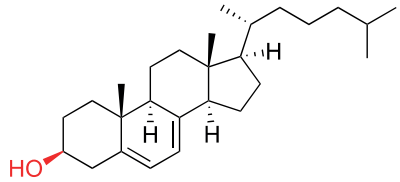
Chol



Desm



7DHC



D_3

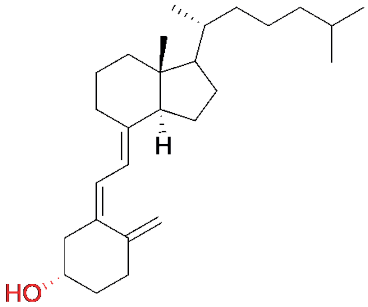


Figure 1. Chemical structures of cholesterol (Chol), desmosterol (Desm), 7-dehydrocholesterol (7DHC) and vitamin D (cholecalciferol, D_3).

Results

Quantification of Glc7DHC, $GlcD_3$ and GlcDesm by LC-MS/MS

Based on our previously developed LC-MS/MS procedure for quantitative detection of GlcChol [6], we developed a LC-MS/MS procedure for quantitative detection of Glc7DHC, $GlcD_3$ and GlcDesm. For this purpose, internal standards, $^{13}C_6$-Glc7DHC and $^{13}C_6$-GlcDesm, were synthesized. This avoids the need for corrections for extraction efficiency, chromatographic behaviour and ionization efficiency during quantification. Lipids were extracted by Bligh and Dyer followed by butanol/water extraction.

For the quantification of Glc7DHC, $GlcD_3$ and GlcDesm in plasma, a calibration curve was performed in plasma by spiking the standard solution of Glc7DHC, $GlcD_3$ and GlcDesm. The calibration curve is: 0 – 0.1 – 0.5 – 1 – 2 – 4 – 10 – 20 – 50 – 100 – 200 nM. As internal standards $^{13}C_6$-Glc7DHC was used for quantification of Glc7DHC and $GlcD_3$ and $^{13}C_6$-GlcDesm for quantification of GlcDesm. A fixed amount of internal standard was added to the sample before extraction and the samples were extracted and measured on the same day. The ratio, area of analyte/area of internal standard, was plotted against the concentration of analyte in spiked plasma (Figure 2A and 2B). For GlcDesm a similar plot was made using the area from the transition of $^{13}C_6$-GlcDesm (Figure 2C).

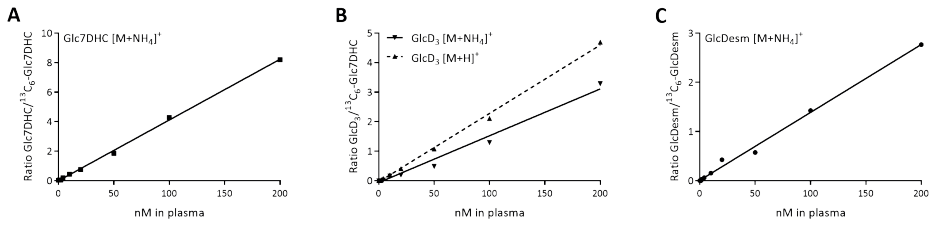


Figure 2. Linearity of Glc7DHC, $GlcD_3$ and GlcDesm in plasma. *A) Glc7DHC absolutely dominant transition $[M+NH4]^+$. B) $GlcD_3$ major transition $[M+H]^+$ and minor transition $[M+NH4]^+$. C) GlcDesm absolutely dominant transition $[M+NH4]^+$. X-axes represents the spiked concentration in plasma (nM), Y-axes represents ratio between analyte and the corresponds $^{13}C_6$-standard.*

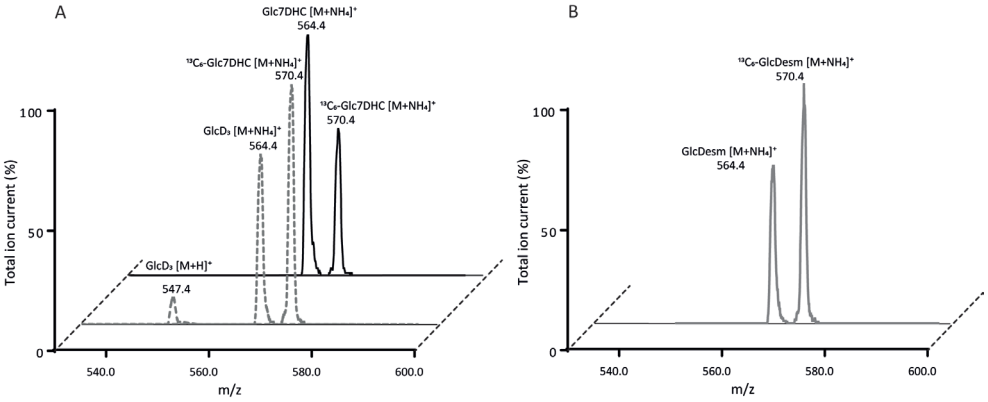
As for GlcChol [6], for all three compounds a linear response was obtained over the entire concentration range (Figure 2 and Table 1). The corresponding limit of detection (LOD) and limit of quantification (LOQ) for the compounds in plasma are given in Table 1. Calculation of the signal-to-noise (SN) was done utilizing the peak-to-peak method.

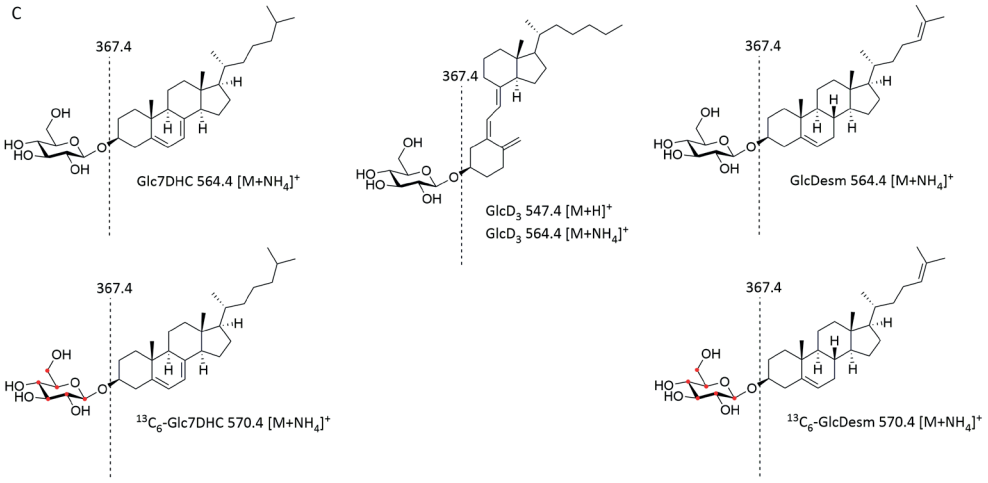
Table 1. Limit of Detection (LOD), Limit of Quantification (LOQ) and corresponding signal-to-noise ratios (S/N).

Compound	R^2	LOD (nM)	S/N	LOQ (nM)	S/N
Glc7DHC	0.999	0.1	3	0.3	10
$GlcD_3$ $[M+H]^+$ $GlcD_3$ $[M+NH_4]^+$	0.998 0.983	0.1	3	0.3	10
GlcDesm	0.995	0.1	5	0.5	10

Mass spectrometric analysis, fragmentation and elution spectrum

Mass spectra were recorded from the newly synthesized unlabelled Glc7DHC, $GlcD_3$ and GlcDesm and $^{13}C_6$- labelled Glc7DHC and GlcDesm, injected as a mixture of 10 µM each (Figure 3A and 3B). Under influence of collision-induced dissociation the compounds lose the glucose moiety, resulting in a product ion of *m/z* 367.4 (Figure 3C). The most abundant species for GlcChol, GlcDesm and Glc7DHC were ammonium adducts, $[M+NH_4]^+$. For D_3 no clear abundant species could be detected. So, both hydrogen adducts, $[M+H]^+$ and ammonium adducts, $[M+NH_4]^+$ are reported. The ^{13}C-isotopes are on the glucose molecule, therefore the daughter fragment of the compounds have the same *m/z* ratio as the not isotope compound. The expected *m/z* values for each compound are presented in Figure 3D. Even though the compounds have the same mass, each compound elutes at a different retention time (Figure 3E and 3F).





D

Compound	Mass	$[M+NH_4]^+$ (Parent)	$[M+H]^+$ (Parent)	Product ion (Daughter)
$^{13}C_6$-Glc7DHC	552.4	570.4	NA	367.4
Glc7DHC	546.4	564.4	NA	367.4
$GlcD_3$	546.4	564.4	547.4	367.4
$^{13}C_6$-GlcDesm	552.4	570.4	NA	367.4
GlcDesm	546.4	564.4	NA	367.4

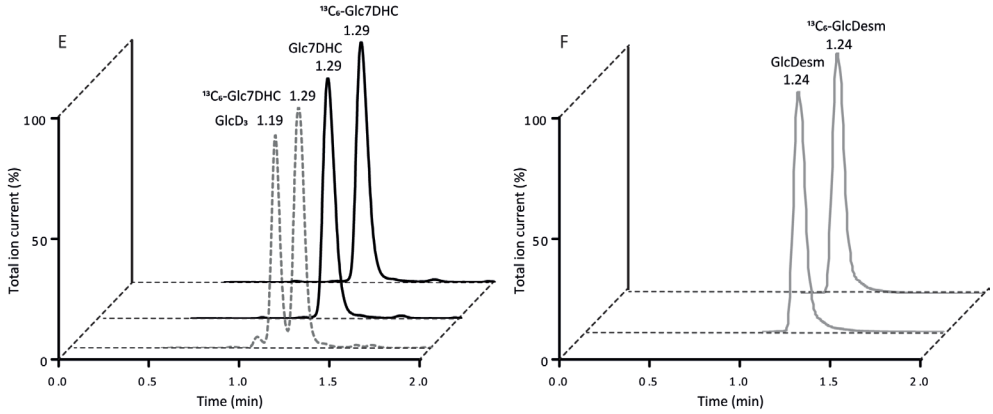


Figure 3. Mass spectrometric analysis. *A) MS-scan of Glc7DHC, GlcD$_3$ and $^{13}C_6$-Glc7DHC. B) MS-scan of GlcDesm and $^{13}C_6$-GlcDesm. C) Fragmentation pattern, the common product ion is 367.4 for all compounds loss of glucose moiety. D) Compounds mass,* m/z *values of parents and daughters. E) Elution pattern of Glc7DHC, GlcD$_3$ and $^{13}C_6$-ß-Glc7DHC. F) Elution pattern of GlcDesm and $^{13}C_6$-ß-GlcDesm.*

Intra/Inter assay variation

The precision of the quantification was determined by intra-assay and inter-assay variations. Figure 4 and Table 2 show the intra and inter-assay variation. The intra- and inter-assay variation was over all compounds on average 15.6% and 20%, respectively (ranges 5.9 – 32.8% for the intra-assay variation and 5.8 – 37.1% for the inter-assay variation. The ranges for Glc7DHC and GlcDesm are acceptable, as Glc7DHC ranges 5.9 – 10.6% and GlcDesm 14.7 – 23.7% for intra-assay variation and 6.2 – 14.1% (Glc7DHC) and 5.8 – 11.4% (GlcDesm) for inter-assay variation. Due to lack of a $^{13}C_6$-GlcD$_3$ internal standards the variations for GlcD$_3$ $[M+H]^+$ and GlcD$_3$ $[M+NH_4]^+$ ranges 8.2 – 32.8% for intra-assay variation and ranges 25.4 -37.1% for inter-assay variation.

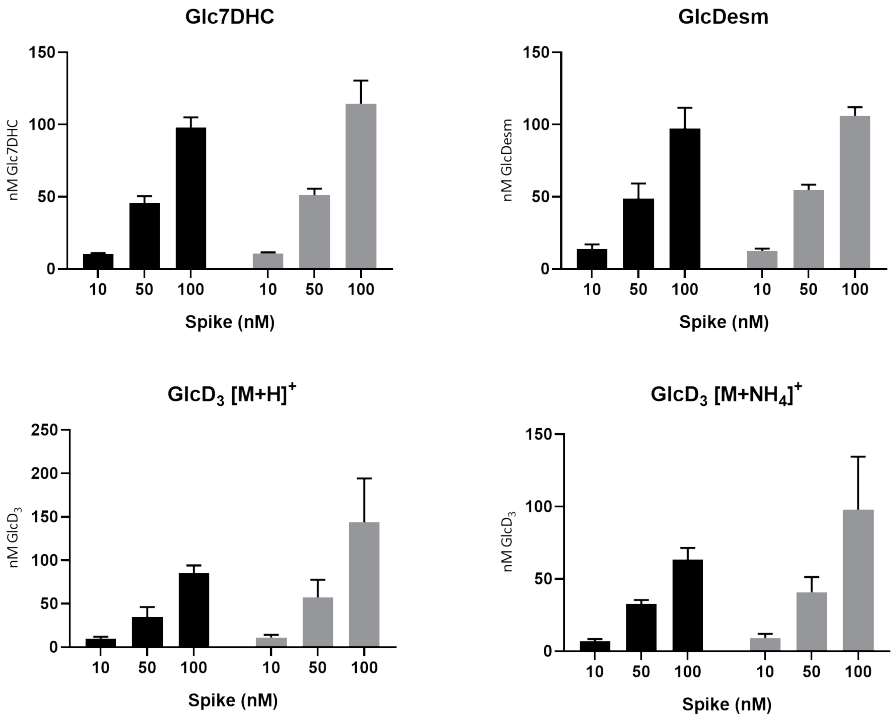


Figure 4. Intra-assay and inter-assay validation. *A) Left bars (black) represent intra-assay and right bars (grey) represent inter-assay validation. All samples were corrected for endogenous present analytes. X-axes represents the spiked concentration (nM), Y-axes represents the found concentration of the analyte in plasma (nM).*

Table 2. Intra-assay and inter-assay variation. In nM (n=5).

		10 nM			50 nM			100 nM		
Compound	**Assay**	**Mean**	**SD**	**CV%**	**Mean**	**SD**	**CV%**	**Mean**	**SD**	**CV%**
Glc7DHC	Intra	10.4	0.6	5.9	45.6	4.8	10.6	98.1	6.9	7.0
	Inter	10.8	0.7	6.2	51.2	4.3	8.3	114.4	16.1	14.1
$GlcD_3$ $[M+H]^+$	Intra	10.0	1.9	19.2	34.7	11.4	32.8	85.2	8.9	10.4
	Inter	11.2	3.0	26.3	57.3	20.2	35.3	143.9	50.4	35.0
$GlcD_3$ $[M+NH_4]^+$	Intra	7.0	1.4	20.3	32.7	2.7	8.2	63.4	8.0	12.6
	Inter	9.2	2.8	30.6	40.9	10.4	25.4	98.1	36.4	37.1
GlcDesm	Intra	13.8	3.3	23.7	48.8	10.4	21.3	97.4	14.3	14.7
	Inter	12.6	1.4	11.4	54.7	3.6	6.5	106.0	6.1	5.8

Carryover

The carryover was determined by injecting twice methanol following the injection of pure standards (0.05 µM and 1 µM) or injection of standards spiked in biological samples. All 0.5 µM injections of pure standards and the standards spiked in biological samples revealed a carryover of zero. The injection of 1 µM pure $^{13}C_6$-Glc7DHC and pure Glc7DHC showed a carryover of ≤0.03% after the first methanol injection, and zero after the second methanol injection. Injection of 1 µM pure $^{13}C_6$-GlcDesm and pure GlcDesm showed a carry-over of ≤0.02% after the first and second methanol injection, and zero after the third methanol injection. Based on these findings injecting methanol twice between each set of GlcDesm samples is advised.

Storage of samples

Storage of skin. Several skin samples of the same healthy person were measured fresh (non-stored, extraction and measurement on same day) and stored at different temperatures: fridge (4°C), freezer (-20°C), freezer (-80°C) for 1.5 month. Lipids were extracted and measured on the same day as samples were taken out of storage. As shown by Figure 5 GlcChol is stable for all conditions, Glc7DHC on the other hand is not. Samples stored at -20°C and -80°C show higher level of Glc7DHC (pmol/g of tissue), indicating that due to storage the structure of other lipids rearranged into Glc7DHC. Detection of Glc7DHC was 60% less for -80°C stored skin compared to non-stored skin (data not shown). We propose to measure the skin samples fresh for the most reliable Glc7DHC measurement.

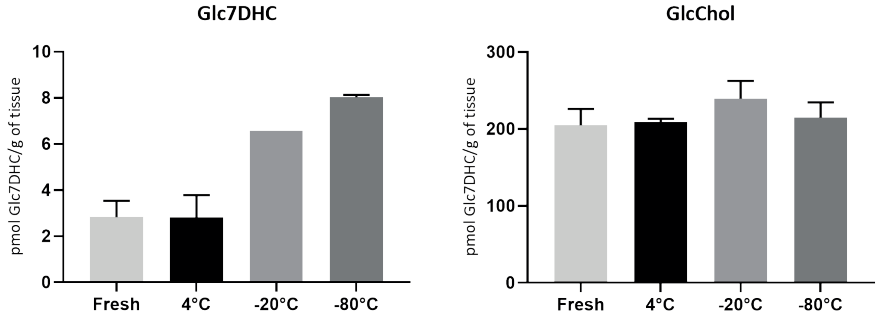


Figure 5. Glycosylated sterols in skin stored for 1.5 month at different conditions. *From left to right bars samples were fresh (non-stored), kept in fridge (4°C), freezer (-20°C) or freezer (-80°C). Measurement of Glc7DHC at -20°C shows no error bar due to sample loss.*

Storage of plasma. Plasma samples were stored for either 10 days at RT, fridge (4°C) and freezer (-20°C) or 14 days at freezer (-20°C) and freezer (-80°C). As shown by Figure 6 for Glc7DHC, GlcDesm, and GlcChol no significant difference in levels were noted, when stored at different conditions. As standard deviations are smallest for storage at -20°C and -80°C, storage at these temperatures is advised. $GlcD_3$ on the other hand seems less stable, and shows a variability between the $[M+H]^+$ and the $[M+NH_4]^+$ transition, especially for storage at -80°C. The spiked concentration of 10 nM was not detectable anymore for the $[M+NH_4]^+$ transition, indicating that the $[M+H]^+$ is the more suitable transition for measuring low concentrations.

Storage of breast milk. Breast milk samples, were stored for 14 days at freezer -20°C and -80°C. As shown by Figure 7 for Glc7DHC, GlcDesm, and GlcChol no difference in levels of the various lipids, when stored at different conditions were noted. $GlcD_3$ shows again less stability and shows, just as in the plasma, variability between the $[M+H]^+$ and the $[M+NH_4]^+$ transition. Again the $[M+H]^+$ is the more suitable transition for measuring low concentrations.

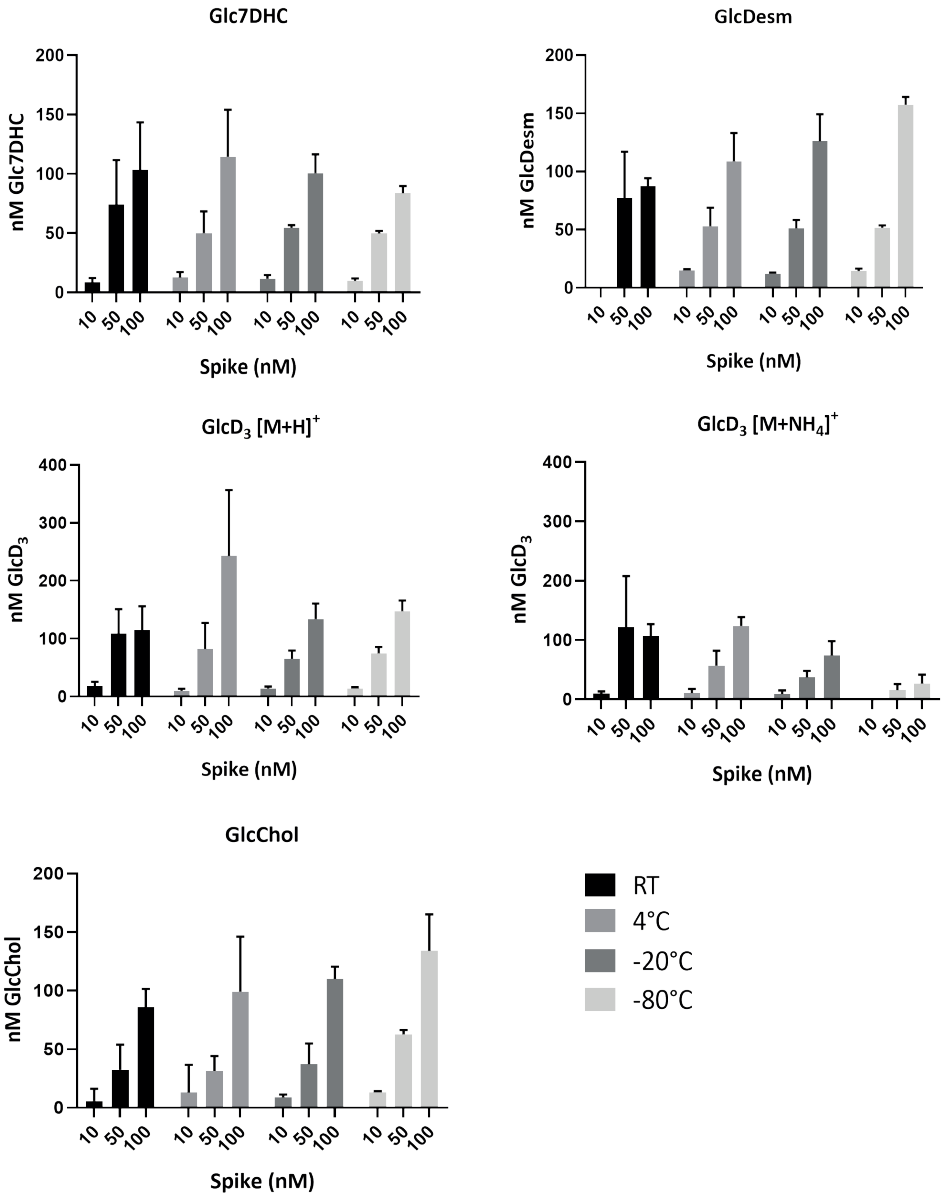


Figure 6. Glycosylated sterols in plasma stored at different conditions. *From left to right bars samples were kept at room temperature (RT), in fridge (4°C), freezer (-20°C) or freezer (-80°C). Presented data were calculated with correction for the appropriate blanks.*

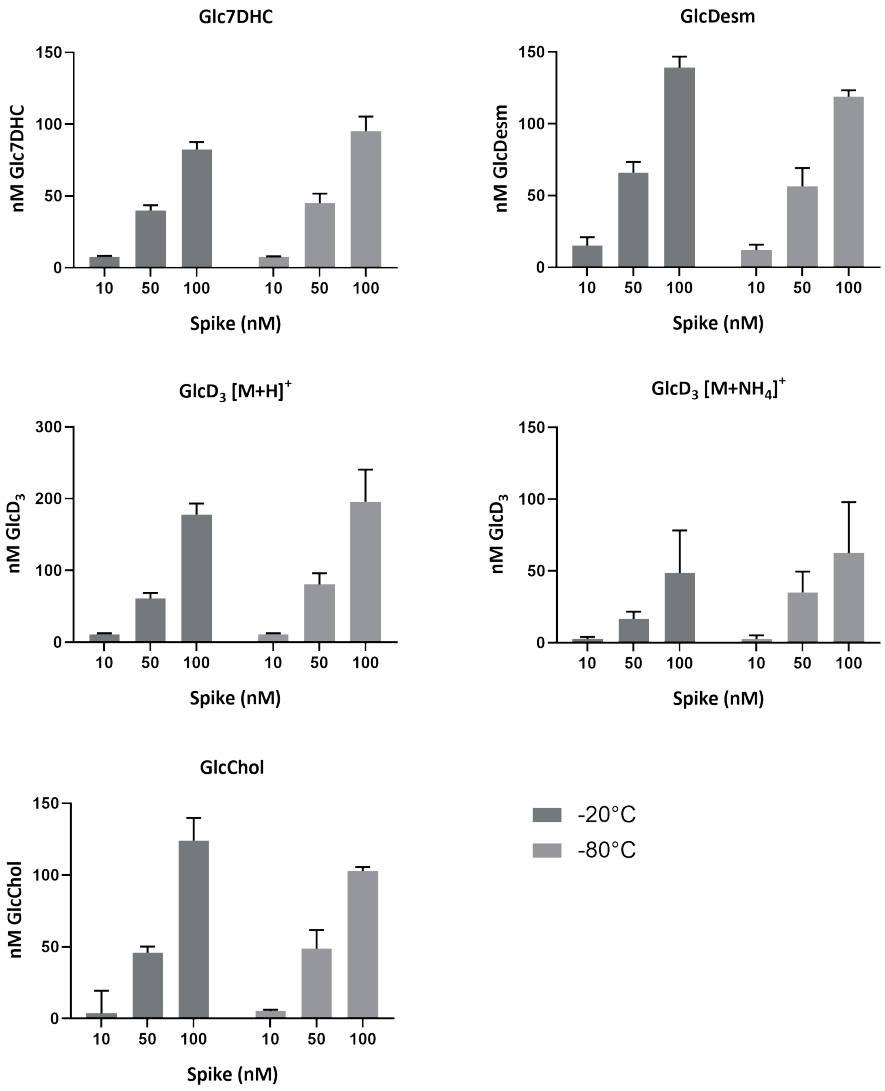


Figure 7. Glycosylated sterols in breast milk stored for 14 days at different conditions. *From left to right bars samples were kept at freezer -20°C or -80°C. Presented data were calculated with correction for the appropriate blanks.*

Impurities

The specific lipid standards were synthesized at the department of Bio-organic Synthesis. Minor impurities, as shown in Table 3, were observed when injecting 3 µL of 0.1 µM of standard on the LC-MS/MS. For all standards, the impurities are within acceptable percentage, with exception of an average 33% (27-39% respectively) impurity of GlcChol in the Glc7DHC, which might have to be purified or resynthesized in the future.

Table 3. Impurities in synthesized standards.

Standard	Compound causing impurity:	Amount of impurity (%)
$^{13}C_6$-GlcChol	GlcChol $^{13}C_6$-GlcDesm	0.2 0.3
GlcChol	GlcDesm	1.1
$^{13}C_6$-GlcDesm	GlcDesm GlcChol	0.5 0.03
GlcDesm	GlcChol	0.1
$^{13}C_6$-Glc7DHC	Glc7DHC $^{13}C_6$-GlcChol	1.6 1.2
Glc7DHC	GlcChol	27-39
$GlcD_3$	GlcChol	0.3

Detection of glycosylated sterols in biological samples

The use of the described LC-MS/MS method allows detection and quantification of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm in several fluids and tissues.

Plasma. Within plasma of ten healthy individuals, five females, five males, ranging in age from 30 of 82 the glycosylated sterols of GlcChol (average 170 nM in plasma), Glc7DHC (average 0.54 nM in plasma) and GlcDesm (average 2.84 nM in plasma) could be detected (Figure 8A). $GlcD_3$ could not be detected. No correlation in relation to age was observed.

Breast milk. Two healthy lactating females donated breast milk for measurement. Female one has been lactating for 2.5 months, female two has been lactating for 21 months. As shown by Figure 8B both GlcDesm and GlcChol could be detected, Glc7DHC and $GlcD_3$ were not detected. The data shows the longer the female lactates, the more the levels of both GlcChol and GlcDesm decrease. For female 2 only traces of GlcDesm were detectable. The decrease is expected as levels of Chol and Desm in breast milk are known to decrease after lactating 30 days postpartum [12]. To confirm the data of Figure 8B a larger cohort is needed. We propose to measure breast milk samples of at least five different females at various times

postpartum, e.g., 1, 2, 3, 6, 12 and 24 months of lactation. This will allow the monitoring of the levels of GlcChol and GlcDesm within the breast milk over time.

Skin. As shown by Figure 8C in skin of three healthy female individuals GlcChol, Glc7DHC and GlcDesm are present. $GlcD_3$ could not be detected. The age of the examined individuals ranges from 21 – to 50 year, and skin colour ranges from white to dark (Table 4). The data implies age dependence for GlcChol and GlcDesm, as levels of patient number 1 and 3 with comparable age are similar. Glc7DHC seems to increase with increase skin pigmentation. In order to confirm this observation a larger cohort of samples is required. We propose at least three different people per skin colour, per age class.

Spleen. The presence of GlcChol and Glc7DHC were detected within both human spleen from healthy control as in tissue from Gaucher disease patients. Levels of both glycosylated sterols are affected by GD, as the levels differ significantly from the control spleens ($P<0.05$).

Table 4. Age and skin colour per patient (female).

Patient nr	Age	Skin colour
1	21	White
2	50	Tanned
3	23	Dark

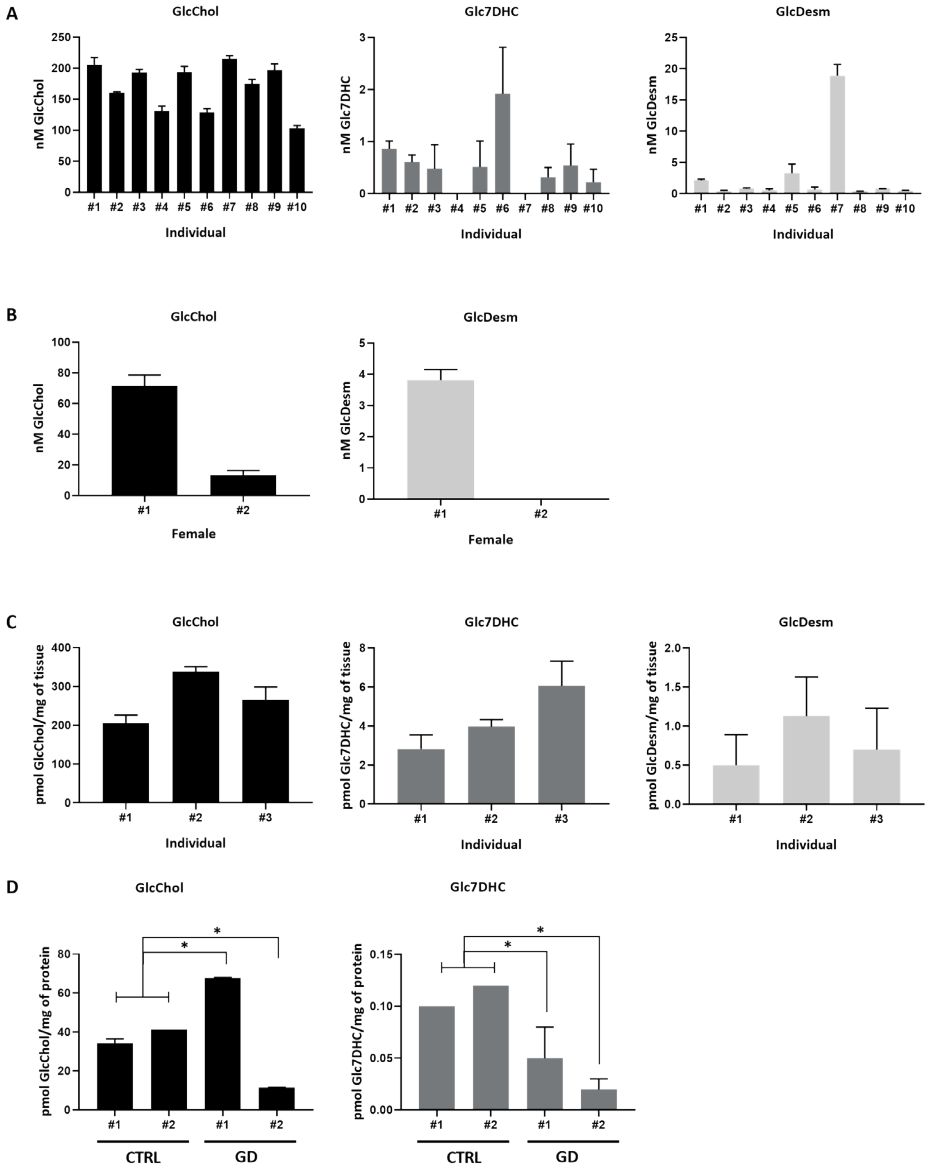


Figure 8. Glycosylated sterols in biological sample.

*A) GlcChol, Glc7DHC and GlcDesm in plasma of 10 healthy individuals. B) GlcChol and GlcDesm in breast milk of two lactating females. For female 2 only traces of GlcDesm could be detected. C) GlcChol, Glc7DHC and GlcDesm in skin of three healthy female individuals. D) GlcChol and Glc7DHC in control (CTRL) and Gaucher Disease (GD) spleen. *P<0.05.*

Discussion

In order to identify and quantify Glc7DHC, $GlcD_3$ and GlcDesm in biological materials we developed LC-MS/MS methods. The methods exploit isotope-labelled internal standards which are chemically identical to the analytes. This allows correction for variations due to extraction, minimizes matrix effects and LC-MS/MS performance. Resulting in an accurate, reproducible and sensitive assay. As no $^{13}C_6$-$GlcD_3$ is available, the precision for determining $GlcD_3$ is less accurate than the determination of Glc7DHC and GlcDesm. The synthesis of $^{13}C_6$-$GlcD_3$ would improve the described method for detection of $GlcD_3$. Furthermore, a $^{13}C_6$-$GlcD_3$ standard could help in tuning the method for a clear dominant transition for $GlcD_3$, either $[M+H]^+$ or $[M+NH_4]^+$, in order to improve the current LC-MS/MS method for measurement of $GlcD_3$. As $GlcD_3$ $[M+H]^+$ has shown to be more accurate, especially for low concentrations, in this thesis measurements for $GlcD_3$ will be performed and reported with the $[M+H]^+$ transition.

Subsequently our data shows stability of the analytes within biological samples such as plasma and breastmilk. The stability of the analytes within skin needs to be further studied. With the presented method, lipids could be extracted from the skin samples, while the skin is not homogenized. As the full skin is not homogenized a spiking experiment as executed for plasma and breastmilk is difficult to perform. So far, homogenization of skin has proven to be difficult. We tested cryogenic grinding with mortar and pestle within liquid nitrogen and mechanical lysis with glass beads in a FastPrep-24 (MP Biomedicals). The skin tissue is rigid and sticky, resulting in non-homogeneous material. A procedure to homogenize the full skin would allow the combination of the LC-MS/MS method with other assays like an ELISA measurement of Chol or 7DHC, or a fluorescence measurement for enzymatic activity.

A second aim of the investigation was to demonstrate the presence of Glc7DHC, $GlcD_3$ and GlcDesm in biological samples. The data shows that GlcChol can be detected in all samples, making it a good positive control. GlcDesm could be detected in breast milk and in low concentration in plasma and skin samples. Glc7DHC is present in plasma, skin and spleens of both healthy and GD affected samples. $GlcD_3$ could not be detected in any of the biological samples.

In conclusion, sensitive LC-MS/MS methods are established to identify and quantify Glc7DHC, $GlcD_3$ and GlcDesm in *in vitro* samples and biological materials. This allows further research of the biological presence and relevance of the glucosylated metabolites.

Experimental procedures

Materials

Plasma samples. AB-control plasma was obtained from EDTA-anticoagulated blood after centrifugation at 1750 g for 10 min and stored at -20°C until further use. Furthermore, ten EDTA plasma samples of random selected healthy individuals were collected by the Erasmus University Medical Centre. In agreement with the Erasmus MC Code of Conduct for responsible use, samples of patients who signed a noncooperation statement for the use of leftover patient material were excluded. The samples were aliquoted, coded and stored at 4°C. Thawed only once for analysis and measured within a week after collecting. All data were analyzed anonymously.

Breast milk. Two healthy lactating females donated their breast milk. One postpartum 2.5 months, one postpartum 21 months. Breast milk was directly extracted and measured for glucosylated metabolites of interest. The remaining breast milk was aliquoted and stored at -20°C until further use.

Skin. Breast skin from cosmetic surgery was obtained from a local hospital and used within 24 h after surgery. All human skin samples were obtained with consent. From the full thickness skin subcutaneous fat was removed with a surgical scalpel. Skin samples were directly extracted and measured for glucosylated metabolites of interest. The remaining skin was aliquoted and stored at -80°C until further use.

Spleen. Human spleens were obtained either as surgical specimens during therapeutic splenectomy or at autopsy. Clinical examination established the phenotype of the subjects. The spleens were stored at -80°C. Later, homogenates were prepared from the frozen material in water.

Pure grade solvents used for LC-MS/MS were 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).

Specific lipid standards. $^{13}C_6$-β-GlcChol [6], GlcChol [6], $^{13}C_6$-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

Synthesis of glucosylated lipid standards. Synthesis of GlcChol and $^{13}C_6$-GlcChol were performed by Ken Kok following published procedures and their spectroscopic data are in agreement with those previously reported [6]. The synthesis of Glc7DHC, $^{13}C_6$-Glc7DHC and $GlcD_3$ was performed by Dr. Patrick Wisse, whereas the synthesis of $^{13}C_6$-β-GlcDesm and GlcDesm was performed by Ken Kok, both at Leiden Institute of Chemistry. The synthetic procedures and their spectroscopic data will be published somewhere else. All the lipid standards were kept frozen at -20°C in the dark.

Quantification of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm by LC-MS/MS. All experiments were performed on a Waters LC-MS instrument (ACQUITY UPLC H-Class coupled to Xevo-TQS-micro) runned by MassLynx 4.1 software, the data analyses was performed by TargetLynx software (Waters Corporation; Milford MA). Analytes were separated on a Acquity BEH C18 reversed-phase column (2.1 x 50 mm. particle size 1.7 µm) (Waters Corporation) by the following eluents: 2-propanol:water 90:10 (v/v) containing 10 mM ammonium formate (Eluent A); methanol containing 10 mM ammonium formate (Eluent B). The instrument settings used for GlcChol measurement are as described earlier [6]. A procedure for quantification of Glc7DHC, $GlcD_3$ and GlcDesm in plasma was developed using spiking of (isotope-encoded) standards, major adducted fragments were identified and the linearity, limit of detection (LOD) and limit of quantification (LOQ) of Glc7DHC, $GlcD_3$ and GlcDesm was established. For the measurement of Glc7DHC, $GlcD_3$ and GlcDesm the same settings were used, with a few adaptations (Table 5). Resulting in individual method for GlcChol, GlcDesm and a combined method for Glc7DHC and $GlcD_3$. These methods apply an isocratic elution of mobile phases, 10% eluent A and 90% eluent B at a flow rate of 0.250 mL/min for a duration of 5.5 min. To prevent system contamination the divert valve of the mass spectrometer was programmed to discard the UPLC effluent before 1.0 min and after 3.0 min. During the run the column temperature and the temperature of the auto sampler were kept at 23°C and 10°C, respectively. With exception for breast milk samples, as here the temperature of the auto sampler was kept at 20°C, to prevent lipid crystallization, which was observed when the auto sampler was at 10°C. For identification and quantification of the detected peaks, direct injection of 1 µM GlcChol, 1 µM GlcDesm, 1 µM Glc7DHC and 1 µM $GlcD_3$ in methanol and the internal standards of 1 µM $^{13}C_6$-β-GlcChol, 1 µM $^{13}C_6$-β-GlcDesm and 1 µM $^{13}C_6$-β-Glc7DHC in methanol were used.

As GlcDesm, Glc7DHC and $GlcD_3$ have the same mass (*m/z* 564.4>367.4 $[M+NH_4]^+$ and *m/z* 547.4>367.4 $[M+H]^+$), the compounds could only be separated based on Retention Time. LC-MS/MS settings were edited to a gradient method for better separation between the peaks of GlcDesm, Glc7DHC and $GlcD_3$ (Table 6). This method applies a mobile-phase gradient elution during 5.5 min run: 0.0 min 5% eluent A and 95% eluent B at a flowrate of 0.1 mL/min; 1.0 min 10% A, 90% B; 2.5 min 10% A, 90% B at a flowrate of 0.2 mL/min; 3.0 min 10% A, 90% B; 4.0 min 5% A, 95% B; 5.5 min 5% A, 95% B at a flowrate of 0.1 mL/min (Supplemental Figure 2). The eluent was diverted to waste between 0.0 - 1.0 min and 3.0 – 5.5 min to keep the source free of contamination.
Calibration curves. Calibration curves for Glc7DHC, $GlcD_3$ and GlcDesm were constructed by adding 0 – 0.1 – 0.5 – 1 – 2 – 4 – 10 – 20 – 50 – 100 – 200 nM of plasma to healthy control plasma. As internal standard 25 µL of 0.1 pmol/uL $^{13}C_6$-Glc7DHC or $^{13}C_6$-GlcDesm was added. Sample were extracted and measured on the same day.

Inter-Intra assay variation. Different concentrations (respectively 10, 50 and 100 nM plasma) of non-labelled compound (Glc7DHC, $GlcD_3$, GlcDesm and GlcChol) were spiked into control plasma of a healthy subject. Samples were extracted in six-fold (five times with the $^{13}C_6$-labelled standards, $^{13}C_6$-GlcChol, $^{13}C_6$-Glc7DHC and $^{13}C_6$-GlcDesm and one without $^{13}C_6$-labelled standards) and measured on the same day to determine the intra-assay variation, and extracted and measured on subsequent days for inter-assay variation.

Storage within plasma. Different concentrations (10, 50, 100 nM) of non-labelled compounds ($GlcD_3$, Glc7DHC, GlcDesm, GlcChol) were spiked into control plasma of a healthy subject. Control plasma of a healthy subject without any spike was also stored. Samples were stored for 10 days at three different conditions: freezer (-20°C), fridge (4°C), and room temperature. After 10 days of storage samples were extracted in four-fold (three times with the $^{13}C_6$-labelled standards, $^{13}C_6$-GlcChol, $^{13}C_6$-Glc7DHC and $^{13}C_6$-GlcDesm and one without $^{13}C_6$-labelled standards) and measured on the same day. Samples were also stored for 14 days at two different conditions: freezer -20°C and freezer -80°C. After 14 days of storage samples were similarly extracted and measured as the samples which were stored for 10 days. Comparison showed for the samples stored at -20°C that storage of either 10 or 14 days revealed no difference (Supplemental Figure 1). Therefore, data for the -20°C was averaged and the -80°C data was included in Figure 6.

Storage within breast milk. Different concentrations (10, 50, 100 nM) of non-labelled compounds ($GlcD_3$, Glc7DHC, GlcDesm, GlcChol) were spiked into breast milk. Breast milk without any spike was also stored. Samples were stored for 14 days at two different conditions: freezer -20°C and freezer -80°C. After 14 days of storage samples were three times extracted in three-fold and measured on the same day.

Table 5. Instrument parameters for individual compound analysis. *Retention Time (RT)*

	GlcChol [6]	**GlcDesm**	**Glc7DHC/GlcD$_3$**
Capillary voltage	3.50 KV	3.50 KV	3.50 KV
Cone voltage	20 V	20 V	20 V
Source temperature	150°C	150°C	150°C
Desolvation temperature	450°C	450°C	450°C
Cone gas	50 L/h	50 L/h	50 L/h
Desolvation gas	950 L/h	950 L/h	950 L/h
Collision voltage	15 V	15 V	15 V
Type	MRM	MRM	MRM
Ion mode	ESI$^+$	ESI$^+$	ESI$^+$
Dwell time	0.100 s	0.165 s	0.040 s
Inter-channel delay	0.005 s	0.005 s	0.005 s
Inter-scan delay	0.005 s	0.005 s	0.005 s
Transitions:	**RT (min.):**	**RT (min.):**	**RT (min.):**
GlcChol	1.37		-
$^{13}C_6$-β-GlcChol	1.37		-
GlcDesm	-	1.24	-
$^{13}C_6$-β-GlcDesm	-	1.24	-
Glc7DHC	-		1.29
$^{13}C_6$-β-Glc7DHC	-		1.29
GlcD$_3$	-		1.19
Smooth method	Mean		
Smooth width	2		

Table 6. Instrument parameters gradient analysis. *Retention Time (RT)*

	GlcChol/GlcDesm/ Glc7DHC/$GlcD_3$
Capillary voltage	3.50 KV
Cone voltage	20 V
Source temperature	150°C
Desolvation temperature	450°C
Cone gas	50 L/h
Desolvation gas	950 L/h
Collision voltage	15 V
Type	MRM
Ion mode	ESI^+
Dwell time	0.054 s
Inter-channel delay	0.005 s
Inter-scan delay	0.005 s
Transitions:	**RT (min.):**
GlcChol	2.66
$^{13}C_6$-β-GlcChol	2.66
GlcDesm	2.40
$^{13}C_6$-β-GlcDesm	2.40
Glc7DHC	2.49
$^{13}C_6$-β-Glc7DHC	2.49
$GlcD_3$	2.31
Smooth method	Mean
Smooth width	2

Extraction method. Before extraction internal standards were added as either 25 or 50 µL of 0.1 µM $^{13}C_6$-β-GlcChol and/or $^{13}C_6$-β-GlcDesm and/or $^{13}C_6$-β-Glc7DHC each in methanol depending on *in vitro* or *in vivo* samples. The sample were subjected to protein precipitation by addition of methanol (MeOH) and chloroform ($CHCl_3$) (2:1. v/v) and shaken for 30 minutes at RT. Afterwards samples were spun down for 10 minutes at 13000 rpm and transferred to a new Eppendorf tube. The protein precipitation was followed by lipid extraction according to Bligh and Dyer [13] by addition of chloroform and water (final volumes: MeOH:$CHCl_3$:H_2O 1:1:0.9, v/v/v). The lower phase (chloroform) was taken to dryness under vacuum at 45°C in a *speed VAC concentrator plus*. The isolated lipids were purified by butanol/water extraction (1:1, v/v) and the upper phase (butanol) was taken to dryness under vacuum at 45 °C in a *speed VAC concentrator plus*. The isolated lipids were resolved in either 75 µL or 100 µL methanol, stirred and sonicated for 30 seconds, stirred once more and centrifuged for 5 minutes at 13000 rpm. Samples are analysed by LC-MS as described above. Volume of injection for *in vitro* samples was 3 µL or 10 µL for *in vivo* samples.

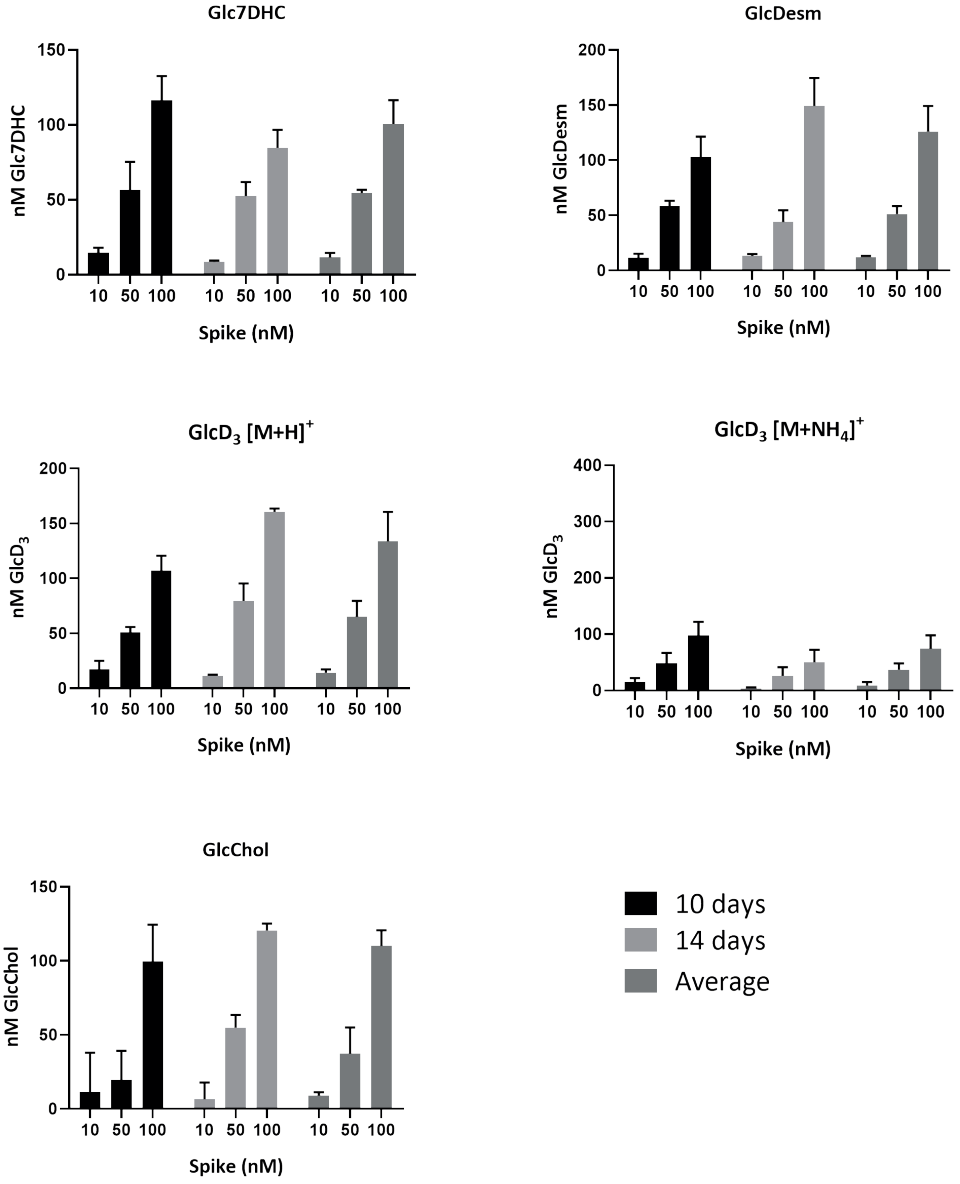
Measurement and quantification of GlcChol, GlcDesm, Glc7DHC and $GlcD_3$ in human plasma, human spleen and human breast milk. The glucosylated sterols were extracted according to the above-described extraction method with a few modifications. Fifty µL of either plasma, homogenised spleen or breast milk was pipetted in an Eppendorf tube (2 mL) and internal standards were added as 25 µL of 0.1 µM $^{13}C_6$-β-GlcChol, 0.1 µM $^{13}C_6$-β-Glc7DHC and 0.1 µM $^{13}C_6$-β-GlcDesm each in methanol. An additional 425 µL methanol and 250 µL chloroform was added. Samples were stirred and left in the dark at room temperature for 30 min, mixed continuously and centrifuged for 10 min at 13000 rpm to spin down protein. The supernatant was transferred to a clean Eppendorf tube and 250 µL chloroform and 410 µL water was added. Samples were stirred shortly and centrifuged for 5 min at 13000 rpm. Lower phase (chloroform) was transferred to a clean Eppendorf tube and the upper phase was washed by addition of 500 µL chloroform. Subsequently, lower phases were pooled and taken to dryness under vacuum at 45°C in a *speed VAC concentrator plus*. The residue was dissolved in 1.2 mL butanol/water (1:1, v/v) and mixed well. After centrifugation (10 min at 13000 rpm), the upper phase (butanol) was transferred to a clean Eppendorf tube and taken to dryness under vacuum at 45°C in a *speed VAC concentrator plus*. Next the residue was dissolved in 75 µL of methanol by mixing and sonication. After centrifugation (5 min for human plasma samples and 10 minutes for human breast milk samples at 13000 rpm), samples were transferred into an MS vial and measured on the LC-MS/MS for determination of GlcChol, GlcDesm, Glc7DHC and $GlcD_3$. As lipid crystallisation was observed in the breastmilk samples after LC-MS measurement, while breastmilk samples were kept at RT before measurement no crystallisation occurred. In order to prevent lipid crystallisation within the LC-MS/MS, breast milk samples were within the auto sampler kept at 20 °C instead of the usually 10 °C. Volume of injection was 10 µL.

Measurement and quantification of GlcChol, GlcDesm, Glc7DHC and $GlcD_3$ in human skin. Full thickness skin was collected and stored over night at 4°C. In the morning of the subsequent day skin was cut in small pieces (100-150 mg) with a surgical scalpel. Internal standards were added as 50 µL aliquots of 0.1 µM $^{13}C_6$-β-GlcChol, 0.1 µM $^{13}C_6$-β-Glc7DHC and 0.1 µM $^{13}C_6$-β-GlcDesm each in methanol. An additional 450 µL methanol and 600 µL chloroform was added. Samples were stirred for 30 seconds alternating 1 min of sonication in a bath sonifier with melting ice, for four times. Subsequently, samples were left in the dark at room temperature for 1.5 h, mixed continuously. Samples were sonicated for 1 min in a bath sonifier with melting ice, twice, alternating 30 seconds of stirring and centrifuged for 10 min at 13000 rpm to precipitate protein. The supernatant was transferred to a clean Eppendorf tube and between 390 µL and 440 µL water, depending on the mg of tissue, was added for Bligh and Dyer extraction. Samples were stirred shortly and centrifuged for 5 min at 13000 rpm. Lower phase (chloroform) was transferred to a clean Eppendorf tube and the upper phase was washed by addition of 600 µL chloroform. Subsequently, lower phases were pooled and taken to dryness under vacuum at 45°C in a *speed VAC concentrator plus*. Butanol/water extraction was performed as described for plasma samples. Next the residue was dissolved in 100 µL of methanol by mixing and sonication. After centrifugation (10 min at 13000 rpm), samples were transferred into an MS vial and measured on the LC-MS/MS for presence of GlcChol, GlcDesm, Glc7DHC and $GlcD_3$. Volume of injection was 10 µL.

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

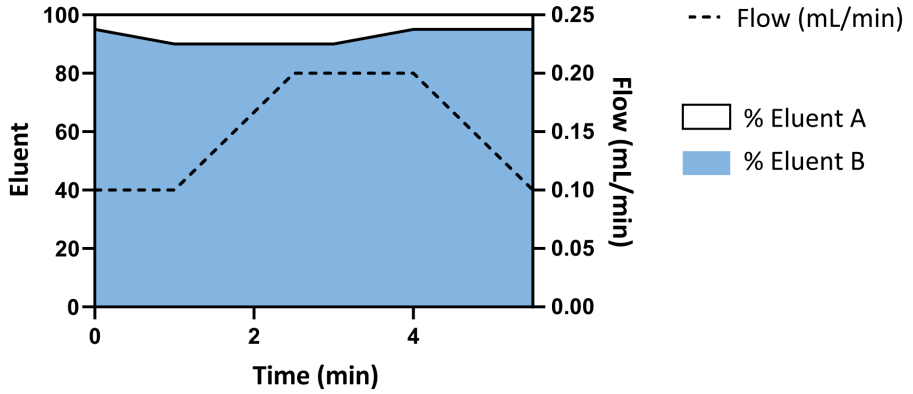
Supplementary information

Supplemental Figure 1. Glycosylated sterols in plasma stored for different days. *Samples were stored in freezer (-20°C). From left to right bars samples were kept in storage for 10 days, 14 days, average of both times of storage. Presented data were calculated with correction for the appropriate blanks.*



Supplemental Figure 2. Gradient elution and chromatogram. *A) Displays the gradient elution of the gradient analysis. B) Displays the chromatograms of the elution of $^{13}C_6$-GlcChol $[M+NH_4]^+$ (RT 2.66), $^{13}C_6$-GlcGlcDesm $[M+NH_4]^+$ (RT 2.40), $^{13}C_6$-Glc7DHC $[M+NH_4]^+$ (RT 2.49), GlcChol $[M+NH_4]^+$ (RT 2.66), $GlcD_3$ $[M+NH_4]^+$ (RT 2.31), GlcDesm $[M+NH_4]^+$ (RT 2.40) and Glc7DHC $[M+NH_4]^+$ (RT 2.49) and $GlcD_3$ $[M+H]^+$ (RT 2.31).*

A



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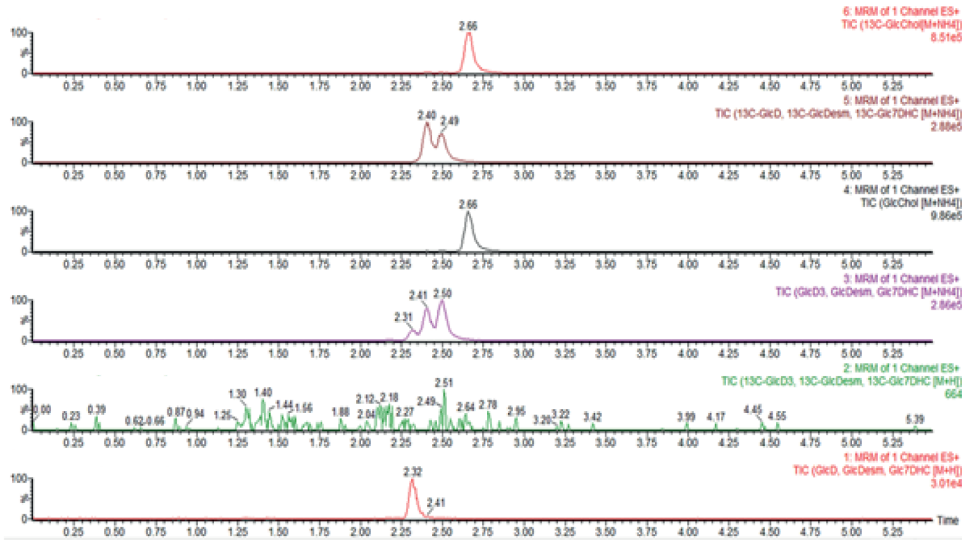

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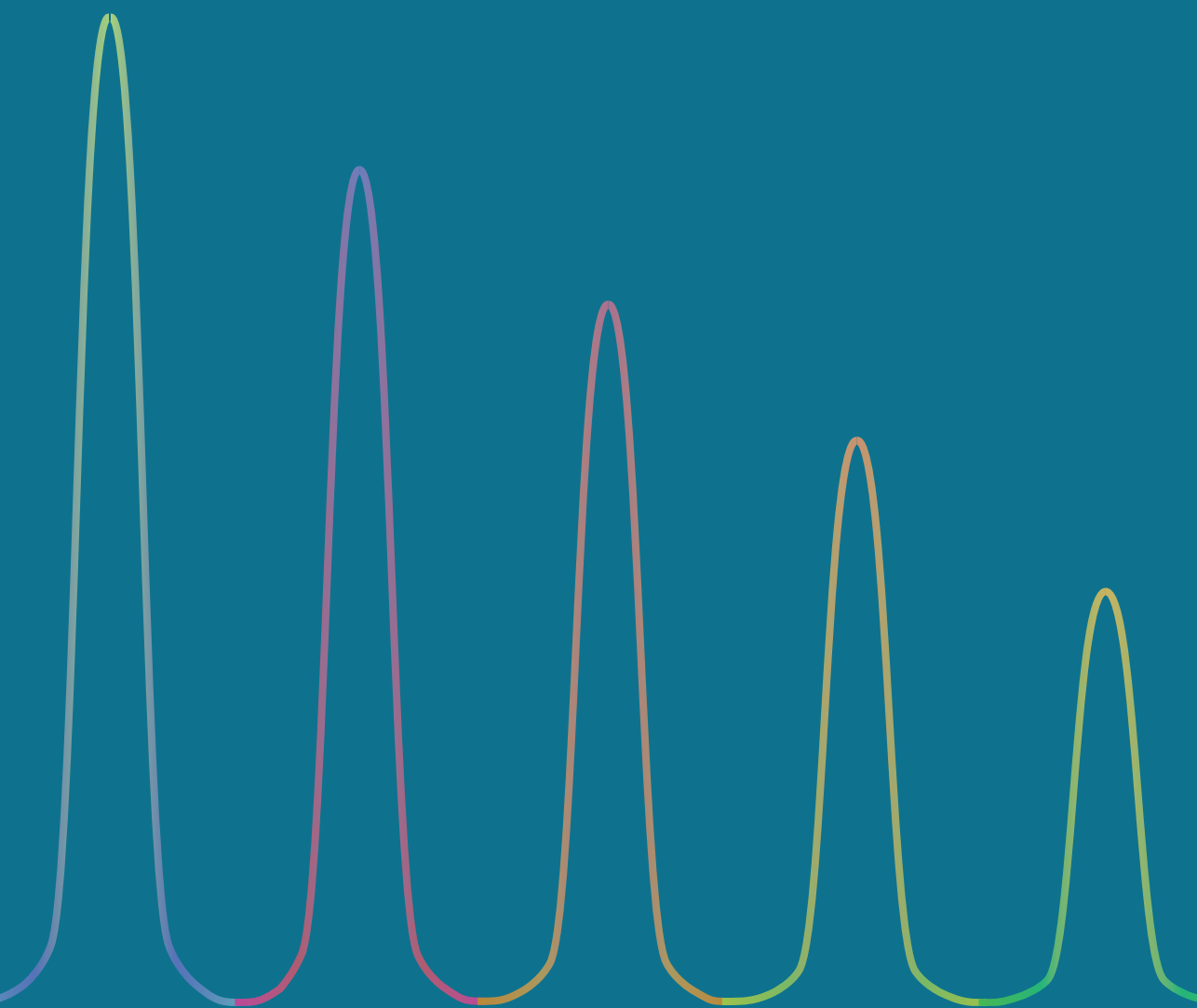

Chapter 3

Formation and degradation of glucosyl-desmosterol

To be submitted in revised form

Chapter 3 - Formation and degradation of glucosyl-desmosterol

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To submitted in revised form

Contributions:
H.N.J.M. : first author, performed described experimental biochemistry and analytical chemistry
K.K. : synthesis of (isotope labeled) GlcChol and GlcDesm
P.W. : (BioSyn, LIC); synthesis (isotope labeled) GlcChol, Glc7DHC and $GlcD_3$
M.M. : advise with LC-MS/MS.
M.A. : supervision of the synthesis of (isotope labeled) GlcDesm
J.M.A. : supervision

Abstract

Desmosterol is an intermediary metabolite with important biological functions. Given the discovery of the transglucosylation of structurally similar cholesterol and 7-dehydrocholesterol, we investigated the potential formation and degradation of glucosylated desmosterol (GlcDesm). During transglucosylation of an acceptor metabolite a glucose is attached via a beta-linkage through transfer from an β-glucoside donor that physiologically is glucosylceramide. The reaction is known to be catalyzed *in vitro* by two cellular retaining β-glucosidases, lysosomal GBA and cytosol-facing GBA2. Our study revealed that glucosylated desmosterol (GlcDesm) is indeed formed *in vitro* by GBA and GBA2 from desmosterol (Desm) and a β-glucoside donor. Importantly, the natural presence of GlcDesm in human spleen could be demonstrated, being elevated in spleen of Gaucher disease patients deficient in GBA. Exceptionally, in spleen of one GD patient no GlcDesm was demonstrable. The accumulation of GlcDesm in GD tissue is explained by the noted ability of GBA to also degrade GlcDesm.

Introduction

Desmosterol (Desm) was first identified in 1956, during research on cholesterol (Chol) in chick embryo's. As Desm closely resembles Chol in was difficult to physically separate from the sterol. Stokes *et al.* gave Desm its name, as they recognized it as $\Delta^{5,24}$-cholestadiene-3β-ol [1, 2]. In the meantime, Bloch was studying the conversion of lanosterol into cholesterol, now known as the Bloch pathway. His work confirmed the existence of Desm [3, 4]. Further work of Stokes *et al.* showed that also rat livers efficiently convert Desm into Chol [5]. In 1964 Roux *et al.* reported on the enzyme responsible for this conversion. While testing the compound tripanol in mice, used for lowering Chol levels, they noticed inhibition of the enzyme 3β-hydroxysterol Δ^{24}-reductase (DHCR24, EC 1.3.1.72), resulting in Desm accumulation [6]. The involvement of DHCR24 in the conversion of Desm into Chol, was confirmed when the first case of desmosterolosis (OMIM #602398) was reported in 1998. As desmosterolosis was a relative unknown disease, it had been, given the similarity in symptoms, mostly considered as Smith-Lemli-Opitz syndrome (SLOS, OMIM #270400) [7]. SLOS is caused by mutations in the 7-dehydrocholesterol reductase (DHCR7) gene, causing storage of 7-dehydrocholesterol (7DHC), another direct precursor of Chol [8, 9]. Ten years after the first report on DHCR24, Croce *et al.* were able to localize DHCR24 on human chromosome 20 [10]. The DHCR24 gene was identified in 2001 by Waterham *et al.* [11], who were the first to identify the human DHCR24 cDNA and confirm the link to the disease desmosterolosis and high levels of Desm. Later research on desmosterolosis confirmed this [12-14].

Besides its role in Chol synthesis, Desm is involved in several other biological processes. Firstly Desm is an abundant lipid in lipid membranes and certain cells, such as spermatozoa [15], astrocytes [16] and fibroblasts [5, 17]. In lipid membranes desmosterol can replace Chol, at first sight without much of influence on the membrane. Mainly proteins inside the membrane seem to be influenced, undergoing conformational changes [17-19]. Complete replacement of Chol by Desm has a dramatic effect in the outer layer of skin, the epidermis. The replacement causes impaired skin barrier function due to increased water loss and skin permeability, resulting in lethal dermopathy in DHCR24-/- mice [20, 21]. In humans lethal dermopathy (OMIM #275210) is known to be caused by a heterozygous mutation in the LMNA gene or by homozygous or compound heterozygous mutation in ZMPSTE24 gene and has as characteristic manifestation thin, tightly adherent translucent skin [22]. As result newborns die within a week [23]. The involvement of DHCR24 in human lethal dermopathy is still elusive. Information of levels of Desm and Chol in this disease are warranted.

Desm is also implicated in cell proliferation [24], cholesterol homeostasis [25], inflammatory responses and pathogen-induced macrophage apoptosis [25, 26], due to its influence on sterol-regulatory element binding proteins (SREBPs) and the Liver-X-Receptor (LXR). Desm is, together with Chol, able to suppress the transcription factor sterol-regulatory element binding protein-2 (SREBP-2), of which DHCR24 is a target [27, 28]. When regulating Chol homeostasis SREBPs cooperate with the nuclear receptor LXR [25]. LXR is activated by the oxysterols 24S,25-epoxycholesterol and 24S-hydroxycholesterol [29], but in macrophage foam cells (macrophages with massive amounts of Chol esters) desmosterol is also successfully activating LXR [30, 31], resulting in anti-inflammatory responses [25]. Lack of LXR results in rapid accumulation of Chol inside the liver, as the response to dietary Chol is hampered [32, 33]. As LXRs are responsible for draining excess sterols and lipids from the Central Nervous System (CNS), absence of LXR causes a defect sterol homeostasis and therefore a sterol overload in the brain. Consequently, motor neurons of the spinal cord start degenerating [34]. Desm is already important during brain development in the fetal phase. In the fetal brain the levels of desmosterol are high in the time before myelination, due to upregulation of DHCR24. The levels of desmosterol drop rapidly in the weeks before birth, as myelination of the brain is finished [35-37].

It is noteworthy that in breastmilk Desm (9%) is besides Chol one of the abundant lipids [38, 39]. Research of Clark *et al.* showed that the average concentration of Desm increased significantly, from 0.6 mg/100 mL at 2 weeks to 1.3 mg/100 mL at 16 weeks postpartum, while the levels of Chol remained rather stable [40]. Along lactation, after 30 days postpartum, Chol and Desm levels start to decrease [39]. In adult human brain the levels of desmosterol are low, due to reduced levels of DHCR24 transcription. Nevertheless, Desm is still the most abundant precursor for Chol formation in brain [37, 41]. It is shown that DCHR24 is abnormal in the brain of Alzheimer's Disease (AD) patients. In specific areas of AD brain, DHCR24 activity in increased, resulting in lower levels of desmosterol and higher levels of Chol compared to control brain [42, 43]. In AD plasma it results in a decrease in the desmosterol/Chol ratio [42], implying that excess Chol is leaving the brain through the blood brain barrier. Increased Chol is known to be a risk factor for atherosclerosis, causing cholesterol-rich deposits in arterial walls [44]. Interestingly AD patients show signs of atherosclerosis, and both diseases share common risk factors [44, 45]. The noted relation between desmosterol levels and atherosclerosis is intriguing. Not only Chol is deposited in arterial walls, but also desmosterol [46, 47]. Under influence of triparanol, an agent which reduces serum cholesterol levels by replacing cholesterol with desmosterol, the amount of deposited desmosterol is increased [47]. So this intervention is not preventing development of atherosclerosis, as thought earlier [48].

Transglucosylation is a modification catalyzed by retaining β-glucosidases. Cells contain two such enzymes, the lysosomal glucocerebrosidase (GBA) and the cytosol-facing GBA2 [49] that both use glucosylceramide (GlcCer) as substrate. GBA is deficient in Gaucher Disease (GD), lysosomal storage disorder presenting with a wide variety of symptoms ranging from hepatosplenomegaly, pancytopenia, coagulation abnormalities, skeletal complications, neurodegeneration and disturbances in skin permeability [50, 51]. As Desm closely resembles Chol, it therefore can be conceived that Desm can also be transglucosylated into GlcDesm.

In the conducted *in vitro* study evidence was obtained for ability of both GBA and GBA2 to transglucosylate Desm. In addition, we show the occurrence of abnormal levels of GlcDesm in GD patients. The potential roles for GlcDesm in GD and other disease conditions are discussed.

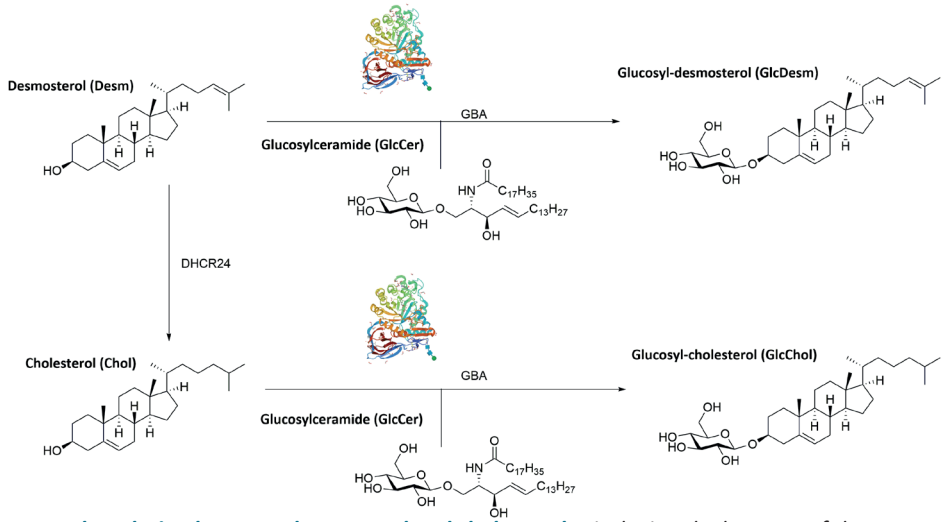

Figure 1 The relation between desmosterol and cholesterol. *Displaying the last step of the Bloch pathway in which desmosterol (Desm) is converted into cholesterol (Chol) by the enzyme 24-Dehydrocholesterol Reductase (DHCR24). Also includes the transglucosylation reaction by the enzyme Glucocerebrosidase (GBA) of Glucosylceramide (GlcCer) into glucosyl-desmosterol (GlcDesm) and glucosyl-cholesterol (GlcChol).*

Results

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

The previous findings on formation of GlcChol [49], but also Glc7DHC and $GlcD_3$ (**Chapter 2** of this thesis) through transglucosylation catalysed by retaining β-glucosidases GBA and GBA2, prompted us to study the possible transglucosylation of Desm. We started with studying the ability of pure recombinant GBA to form GlcDesm. Recombinant GBA was incubated with 4MUGlc (3.7 mM) as glucose donor and Desm (0.3 mM) or Chol (0.3 mM) as acceptor, for 1 hour in 150 mM McIlvain buffer (pH 5.2). The reaction was supplemented with Triton X-100 (0.1% v/v), sodium taurocholate (0.2% w/v) and bovine serum albumin (0.1% w/v). By LC-MS/MS the formation of glucosylated Desm and Chol was monitored.

Subsequently, we studied the GlcDesm formation in comparison to the GlcChol formation by rGBA. By varying time and pH optimal conditions for the tranglucosylation reaction were determined. rGBA produces GlcDesm and GlcChol over time from both glucose donors, 4MUGlc and GlcCer (Figure 2A). In accordance with the previous results for the pH optimum of the generation of the glucosylated metabolites, the optimal pH was found to be between pH 4.0 and pH 5.0 (Figure 2B).

Next, we investigated the ability of the cytosol-facing GBA2 to generate GlcDesm. For this, we incubated homogenates of cells overexpressing GBA2 with Desm and 4MUGlc or GlcCer as glucose donors and analysed formation of GlcDesm by LC-MS/MS. By pre-treating the homogenates with an irreversible GBA inhibitor, adamantyl-cyclophellitol (ME656), the activity of GBA was excluded. Formation of the glucosylated metabolites by GBA2 in the homogenates was observed, with an optimal pH around pH 6.0 (Figure 2C, D). Of note, when homogenates were incubated with Desm without additional glucose donor (4MUGlc or GlcCer) hardly any GlcDesm was detected.

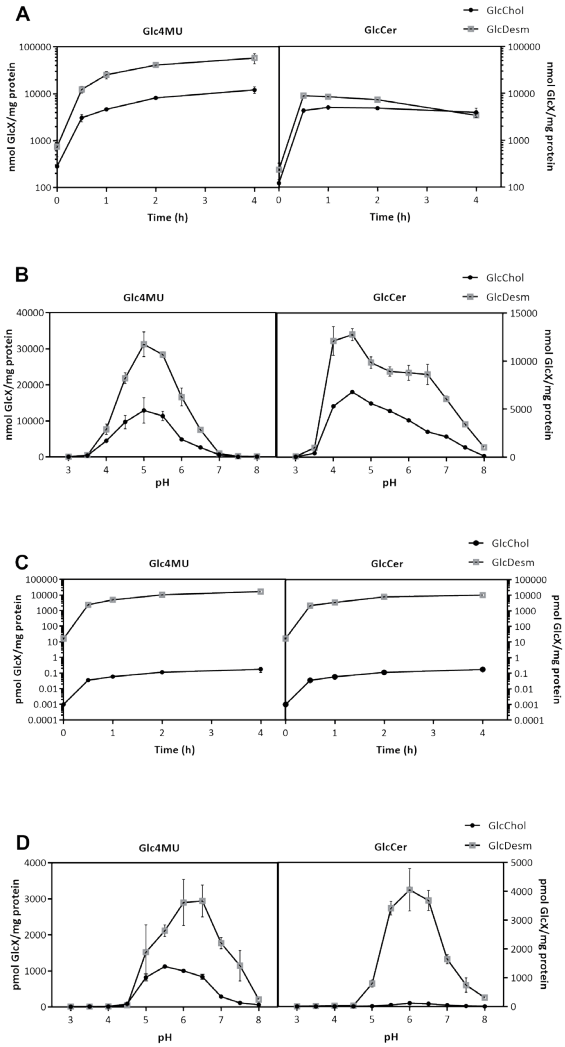

Figure 2. In vitro formation of GlcDesm. *A) rGBA was incubated for varied times with cholesterol (Chol) or desmosterol (Desm) in the presence of 4-methylumbelliferyl ß-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer) donor. Formation of GlcChol and GlcDesm was measured by LC-MS/MS (nmol/mg protein).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

So far, no transglucosylation of Desm by the broad-specific beta-glucosidase GBA3 has been detected (data not shown).

The experiments above were repeated at optimal pH for GBA (pH 5.2) as well as GBA2 (pH 5.8), while measuring both transglucosylation and hydrolase activities. We measured GlcDesm and GlcChol by LC-MS/MS and 4-methylumbelliferone (4MU) with a LS-55 Fluorescence spectrometer over time, resulting in a ratio between transglucosylation and hydrolase activities (pmol GlcX/nmol 4MU) (Figure 3). For rGBA, the formation of both products remained constant over time. On the other hand, GBA2 shows a stable formation of GlcChol over time, but prominent GlcDesm formation increasing over time.

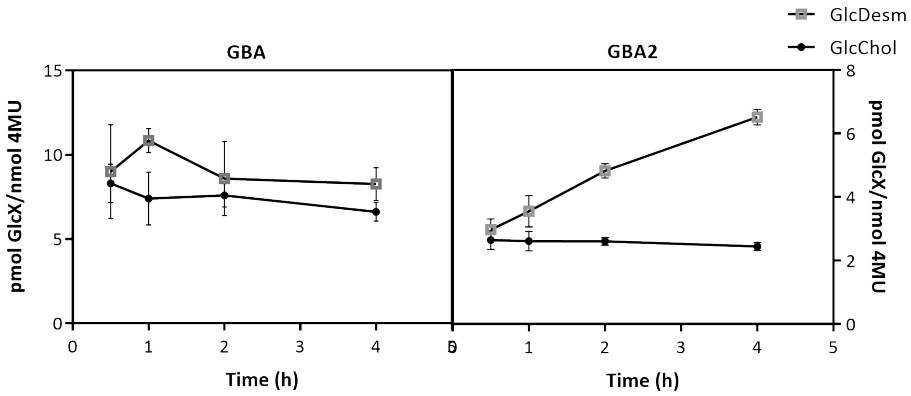

Figure 3. Ratio Transglucosylation and hydrolase activities. *rGBA was incubated for varied times with cholesterol (Chol) or Desmosterol (Desm) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc). 4MU-emitted fluorescence (nmol 4MU/mg protein) was measured with a LS-55 Fluorescence spectrometer (PerkinElmer) using λEX 366 nm and λEM 445 nm. Formation of GlcChol and GlcDesm 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).*

Next, degradation of pure GlcDesm (4 µM) by rGBA and GBA2 was determined by the monitoring of its reduction with LC-MS/MS (Figure 4). For both rGBA and GBA2 we observed a rapid time-dependent degradation of GlcDesm upon incubation at 37°C.

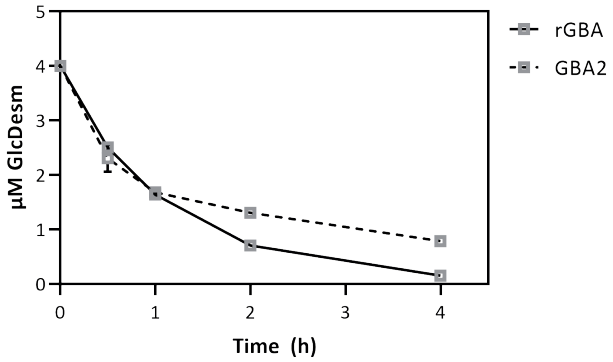

Figure 4. Degradation of GlcDesm. *A) Either rGBA or GBA2 was incubated for varied times with 4 µM GlcDesm. Degradation of GlcDesm was measured by LC-MS/MS (µM) and corrected for amount of protein.*

Measurement of GlcChol and GlcDesm in GD spleen

We investigated human spleen for the presence of GlcDesm and GlcChol, by LC-MS/MS. For quantification internal standards $^{13}C_6$-GlcChol and $^{13}C_6$-GlcDesm were used. Figure 5 shows GlcChol and GlcDesm detection in human control spleen as well as in human GD spleen. Levels of GlcDesm are relatively low as compared to levels of GlcChol. In the spleens of type 1 GD patients elevated levels of both GlcChol and GlcDesm were observed. Exceptional was patient GD3, in whom no GlcDesm was detected.

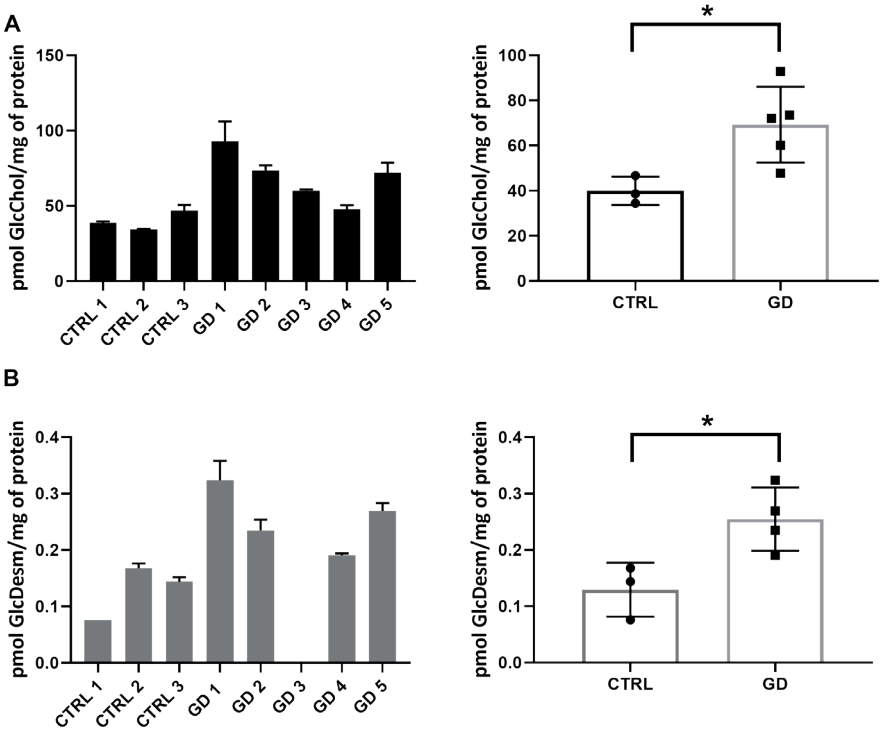

Figure 5. Levels of GlcChol and GlcDesm in GD spleen. *LC-MS/MS analysis of GlcChol and GlcDesm occurrence in human control spleens and human GD spleens. Errors bars are standard deviation of technical duplicates. Data were analysed by unpaired Student's t-test. P values <0.05 were considered significant (*P<0.05). In order to show the significant elevation of GlcDesm in GD patients, the data of patient GD3 was excluded from panel B (right figure).*

Discussion

A sensitive method for measuring GlcDesm was developed (**Chapter 2** of this thesis). With this method we could demonstrate that Desm is an excellent acceptor for transglucosylation. rGBA handles Desm similar to Chol, while GBA2 handles Desm even better than Chol.

When considering the obtained data on *in vitro* formation, hydrolysis, transglucosylation of both GlcDesm and GlcChol and degradation of GlcDesm, the preference of GBA2 for Desm transglucosylation is striking. rGBA is able with both Glc4MU and GlcCer as sugar donor to rapidly form both GlcDesm and GlcChol, with no significant preference for either Desm or Chol. While GBA2 clearly prefers Desm over Chol as acceptor for the transglucosylation reaction. Implying that at lower concentrations of Desm, compared to Chol, similar levels of GlcDesm compared to GlcChol can be reached. This is partly due to the increasing GlcDesm formation by GBA2, a phenomenon that is not observed for rGBA.

It appears that under normal conditions GBA promotes degradation of a glucosylated compound, while GBA2 favours the formation of such glucosylated compounds, with GlcCer as natural sugar donor. As GBA2 prefers the formation of GlcDesm over GlcChol, it might prohibit extra Chol formation via DHRC24. Research of Spann [25] suggest that excess cholesterol inhibits DHCR24 activity, promoting increased endogenous Desm. This feedback loop, causes Desm to interact with LXRs and inhibits SREBPs resulting in inhibition of cholesterol biosynthesis, increase in cholesterol efflux and decrease in proinflammatory gene expression. This response is relevant for atherosclerosis in which plaques in the vessel walls of arteries, impair arterial function [52]. A main reason for this is excess Chol, with an inflammatory response. As marker low plasma high-density lipoprotein cholesterol (HDL-c) is recognized. The response of macrophages to this, is to turn into macrophage foam cells, full of modified lipoproteins [25]. Excess levels of Chol and the inhibition of inflammatory response are in normal conditions countered by an appropriate homeostatic response in which Desm is generated during foam cell formation. Desm as dominant LXR ligand, triggers the discussed feedback loop [25]. Interestingly Gaucher type 1 patients with prominent Gaucher cells show abnormal low levels of HDL-c, but they have no increased risk on atherosclerosis [53]. This suggests that their disbalance in GBA and GBA2 function protects them against atherosclerosis. We suspect that the balance between Chol, Desm, GlcChol and GlcDesm are of high importance in relation to atherosclerosis development.

The ability of both GBA and GBA2 to form GlcDesm, makes it reasonable to think that desmosterolosis patients, who have increased levels of Desm, are prone to develop high levels of GlcDesm as well [7]. One requirement for this is the availability of the sugar donor GlcCer. Increased GlcCer is known for GD as well as for SLOS [49, 54, 55]. Whether levels of GlcCer are elevated within

desmosterolosis patients is not known. Some clinical symptoms, like specific facial features, skeletal problems, affected neurologic central nervous system and growth problems, manifest in desmosterolosis and SLOS and are reminiscent in GD (Supplemental Table 1). Based on this investigation of glucosylated forms of Chol and Desm within these patients might be of interest.

Another clinical feature that deserves consideration is impairment in skin barrier function. This is a phenomenon that is not observed in desmosterolosis patients, but it has been well described for severe Gaucher type 2 patients (OMIM #230900), who presented at birth with a collodion baby phenotype (OMIM #608013) [56-58]. Furthermore, impaired skin barrier function and lethal dermopathy was observed within DCHR24-/- mice [20, 21]. For Gaucher type 2 patients the skin problems seem to be related to elevated levels of GlcCer and decreased epidermal glucocerebrosidase activity [56-59]. On the other hand, desmosterolosis patients, who have high levels of Desm and low levels of Chol display no skin problems. Possibly the ratio changes between present Desm, Chol, but also GlcDesm and GlcChol in the epidermis might play a crucial role. From preliminary data we observed that GlcDesm was not measurable in healthy skin (data not shown), while GlcChol and GlcCer are present [60, 61]. This raises the question what the levels of these metabolites are in patients with demosterolosis, GD2 and lethal dermopathy patients.

The final consideration is that the balance between glucosylated and non-glucosylated compounds is crucial in disease development. Further research of glucosylated compounds within diseases such as desmosterolosis, but also SLOS and GD might help unravel the influence of specific levels of specific glycosylated or non-glycosylated compounds on symptom development. This new view might open up new ways and thoughts on treatments.

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Supplementary information

Supplemental Table 1. Overlap in disease symptoms for Demosterolosis, Smith-Lemli-Opitz syndrome (SLOS) and Gaucher Disease (GD).

		Desmosterolosis	SLOS	Gaucher Disease			
				Type I	Type II	Type III	Perinatal Lethal (collodion baby)
OMIM		#602398	#270400	#230800	#230900	#231000	#608013
Malacards ID		DSM002	SMT004	GCH015	GCH016	GCH017	GCH018
Facial features	Anteverted nares	**X**	**X**				**X**
	Micrognathia	**X**	**X**				**X**
	Microcephaly	**X**	**X**				**X**
	Low-set ears	**X**	**X**				**X**
	Strabismus	**X**			**X**		**X**
Skeletal	Osteosclerosis	**X**		**X**		**X**	
Neurologic Central Nervous System	Seizures	**X**	**X**		**X**	**X (myoclonic)**	**X**
	Spasticity	**X**			**X**		
	Learning disability	**X**	**X**	**X**			
Growth	Failure to thrive	**X**	**X**		**X**		
	Short stature	**X**	**X**	**X**		**X**	

Experimental procedures

Materials

The following pure grade chemicals were used: cholesterol and desmosterol from Sigma-Aldrich (St Louis, MO, USA), D-glucosyl-β-1,1'*N*-oleoyl-D-*erythro*-sphingosine (C18:1-GlcCer) Avanti Polar Lipids (Alabaster, AL, USA), 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) purchased from Glycosynth™ (Winwick Quay Warrington, Cheshire, England), sodium taurocholate from (EMD Millipore Corp, Billerica MA, USA). The department of Bio-organic Synthesis (University of Leiden, The Netherlands) has synthesized the GBA1 inhibitor ME656 as described earlier [62].

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

For LC-MS/MS the following pure grade solvents were used: ethanol was 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. The surgical specimens of human spleens were obtained during therapeutic splenectomy or at autopsy. By clinical examination phenotype of the subjects were determined. Consent was obtained for research use. The obtained surgical specimens of spleens were stored at -80°C. In water homogenates were prepared from the frozen materials.

Specific lipid standards. $^{13}C_6$-β-GlcChol [49], GlcChol [49], $^{13}C_6$-β-Glc7DHC, Glc7DHC, GlcD3, $^{13}C_6$-β-GlcDesm and GlcDesm were synthesized at the Bio-organic Synthesis department (Leiden University, The Netherlands). The full description of the syntheses of $^{13}C_6$-β-GlcDesm and GlcDesm will be described separately.

Methods

In vitro assay of hydrolase activity. As described earlier [63], for GBA activity 3.7 mM 4MU-β-Glc was incubated either with recombinant enzyme 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). For GBA2 activity a lysate of cells overexpressing GBA2 was incubated with 3.7 mM 4MU-β-Glc in 150 mM McIlvaine buffer, pH 5.8 [64]. Addition of excess NaOH-glycine (pH 10.3) was used to stop the reaction and 4MU-emitted 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. The assays were performed as described earlier [49]. Transglucosylation activity of the enzymes GBA and GBA2 were determined with recombinant GBA and lysates of HEK293T cells overexpressing GBA2. As glucose donors, with final concentrations in the reaction of 100 µM C18:1-GlcCer or 2.8 mM 4MUGlc were used. As acceptors with a final concentration in the reaction of 0.3 mM Chol or Desm were used. The samples for measurement of GBA transglucosylation 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). The samples for measurement of GBA2 transglucosylation contained 40 µL of homogenate of GBA2 overexpressing cells, to which was added 10 µL of 50 nM ME656 (GBA1 specific inhibitor) and McIlvain buffer (citrate-phosphate buffer, pH 5.8). Transglucosylation reactions were incubated for 1h at 37°C. The reaction was terminated by putting samples on ice. Hydrolase activity was measured in parallel and the residual sample was subjected to Bligh and Dyer [65] lipid extraction (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-GlcDesm 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.

Degradation of GlcDesm. The monitoring of degradation over time was performed with 4 µM of GlcDesm either with 40 µL recombinant GBA diluted 1:1000 in 25 mM KPI buffer (pH5.2) supplemented with Triton X-100 (0.1% v/v) or with 40 µL of homogenate of GBA2 overexpressing cells, to which was added 10 µL of 50 nM ME656 (GBA1 specific inhibitor) and McIlvain buffer (citrate-phosphate buffer, pH 5.8). For LC-MS/MS analysis all samples were prepared as described previously for GlcChol [49] and measured by LC-MS/MS for GlcDesm levels.

Measurement and quantification of GlcChol and GlcDesm in spleen. Both GlcChol and GlcDesm were extracted from spleen. 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-GlcDesm each in methanol, were added. The procedure of Bligh and Dyer and butanol/water clean-up were continued as described earlier [49].

Protein determination. Protein was measured using the Pierce BCA Protein Assay kit (Thermo Scientific). Absorbance was measured in EL808 Ultra Microplate Reader (BIO-TEK Instruments Inc.) at 562 nm.

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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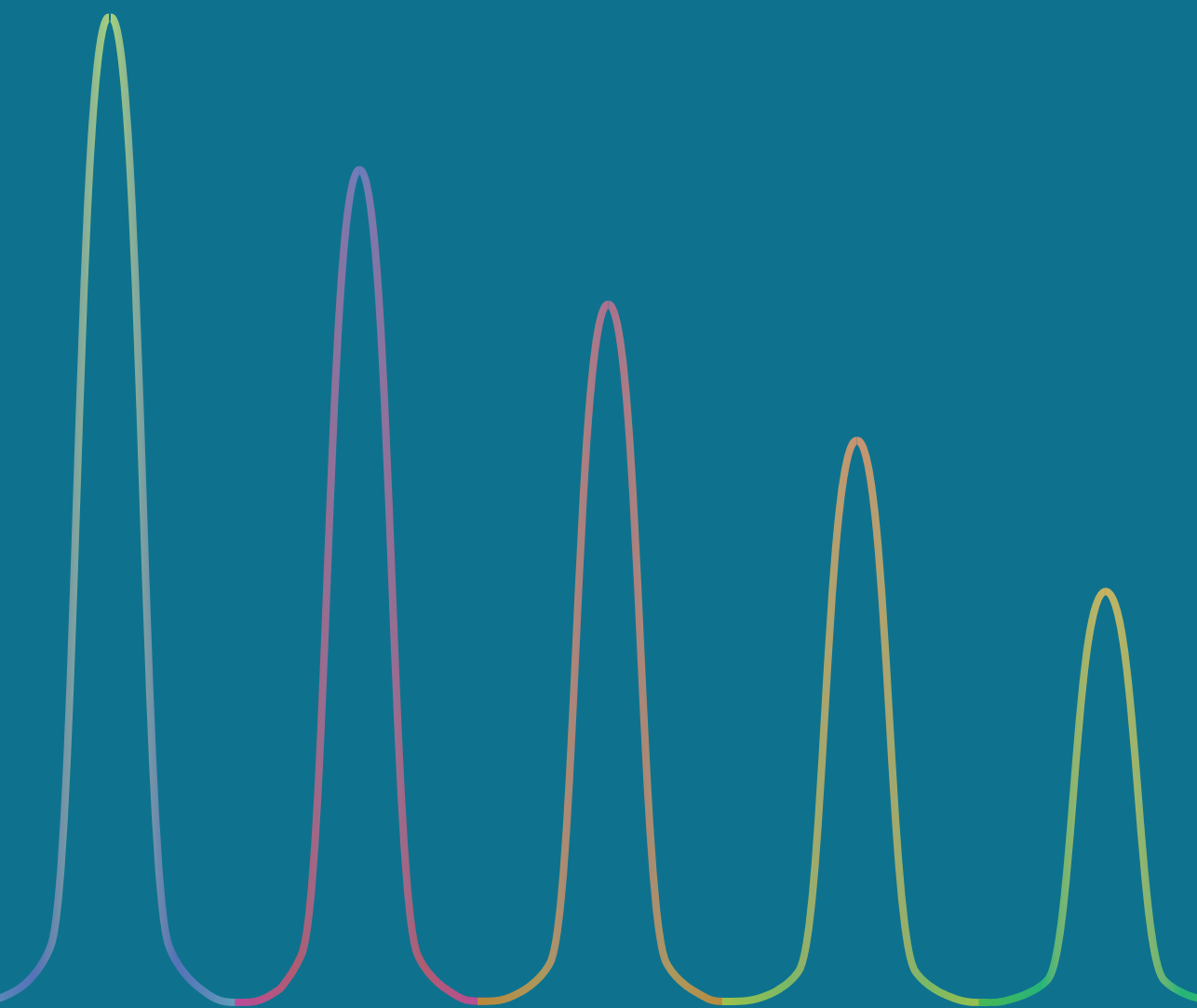

Chapter 4

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.

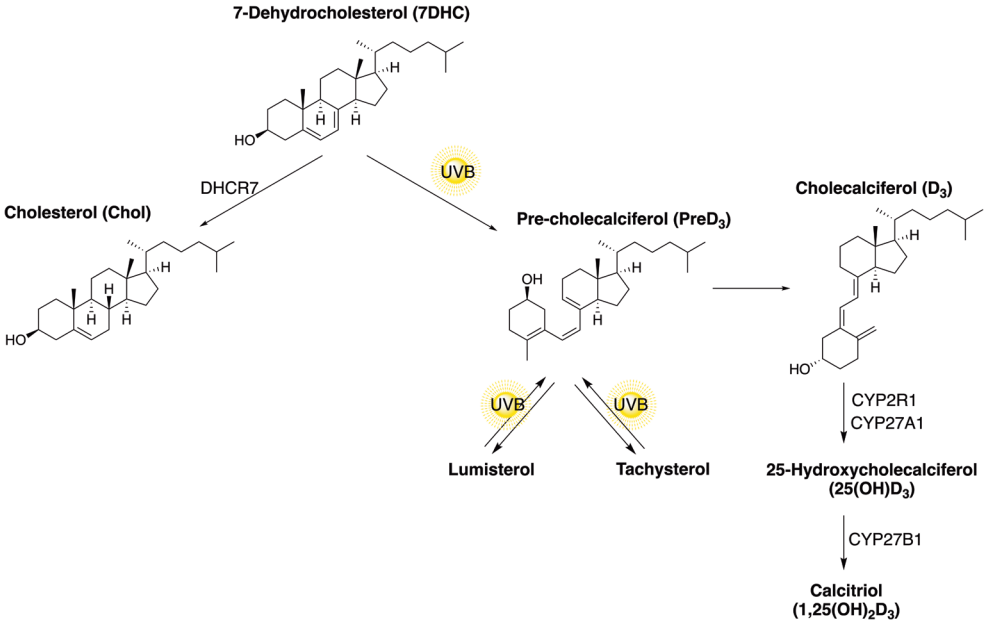


Figure 1. The fate of 7-dehydrocholesterol (7DHC). *Showing involvement of 7DHC in both the cholesterol pathway and the vitamin D_3-pathway. Cholesterol pathway: 7DHC is conversed into cholesterol (Chol) by the enzyme DHCR7 (EC 1.3.1.21). Vitamin D_3-pathway: the first reaction steps from 7DHC to D_3 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.

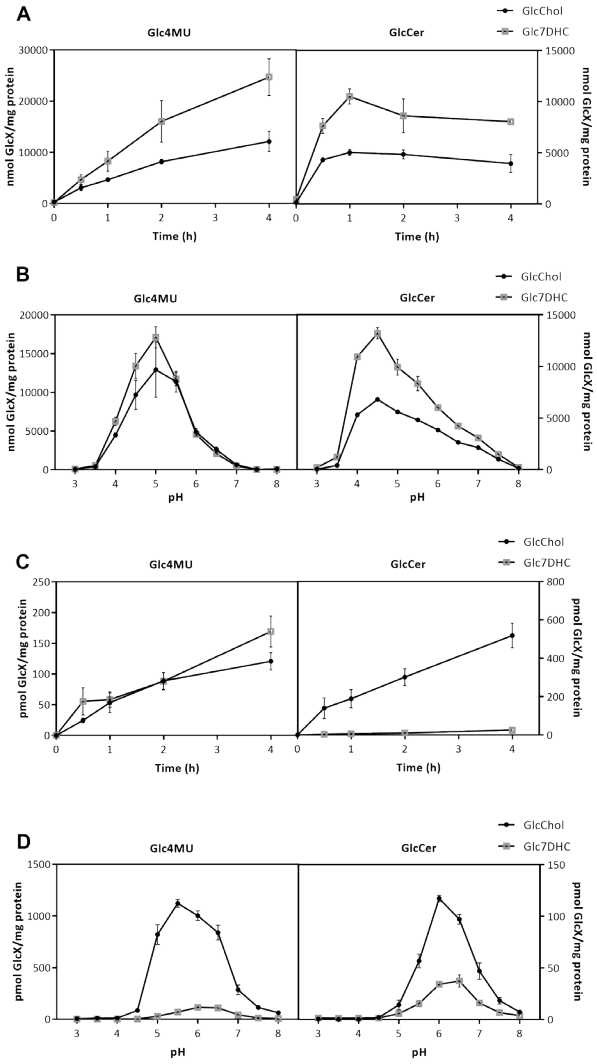


Figure 2. In vitro formation of Glc7DHC. *A) rGBA was incubated for varied times with cholesterol (Chol) or 7-dehydrocholesterol (7DHC) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer) donor. Formation of GlcChol and Glc7DHC was measured by LC-MS/MS (nmol/mg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

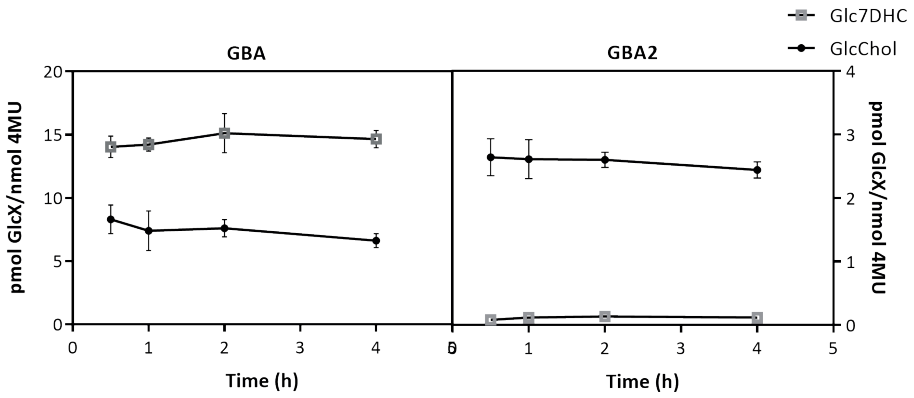


Figure 3. Ratio Transglucosylation and hydrolase activities. *rGBA was incubated for varied times with cholesterol (Chol) or 7-dehydrocholesterol (7DHC) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc). 4MU-emmitted fluorescence (nmol 4MU/mg protein) was measured a LS-55 Fluorescence 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.

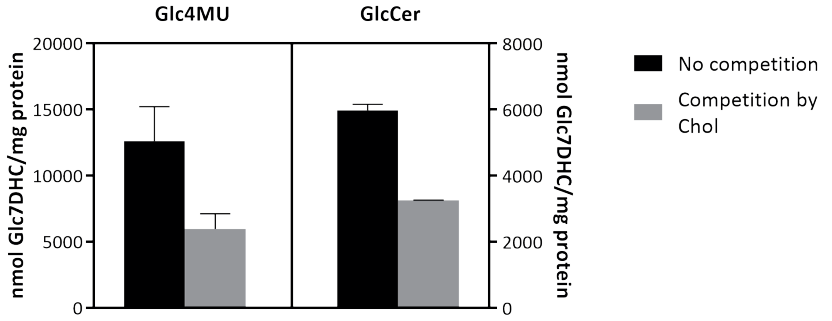


Figure 4. Competition of 7DHC and Chol for transglucosylation. *rGBA was incubated for 1 hour with 7DHC (0.3 mM) only or 7DHC (0.3 mM) and Chol (0.3 mM) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer). Formation of Glc7DHC was measured by LC-MS/MS (nmol/mg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

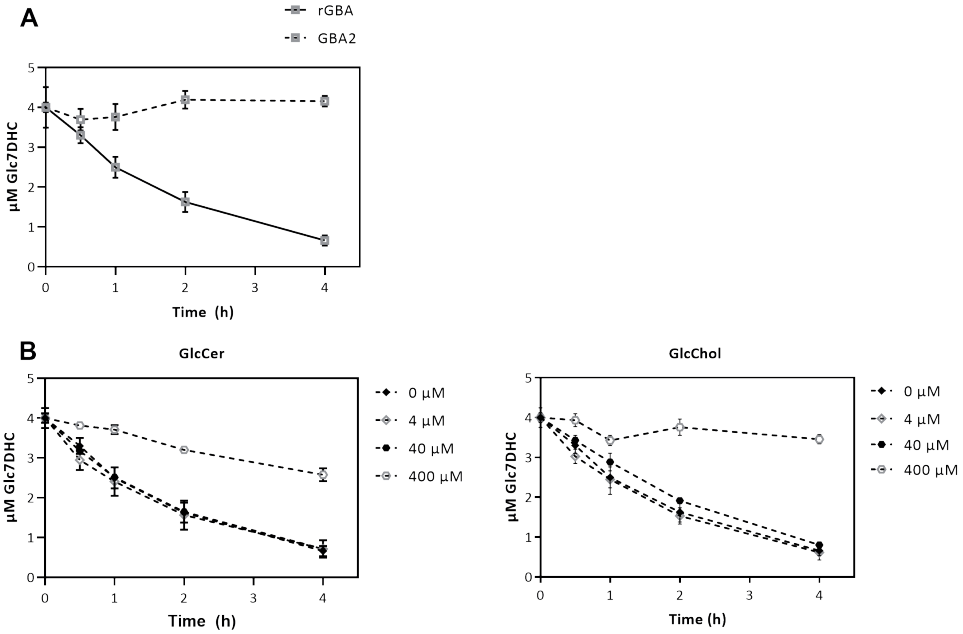


Figure 5. Degradation of Glc7DHC. *A) Either rGBA or GBA2 was incubated for varied times with 4 µM Glc7DHC. B) Influence of GlcCer or GlcChol, in concentrations of 4, 40 or 400 µM, on the degradation of Glc7DHC by rGBA are shown. Degradation of Glc7DHC was measured by LC-MS/MS (µM) and corrected for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

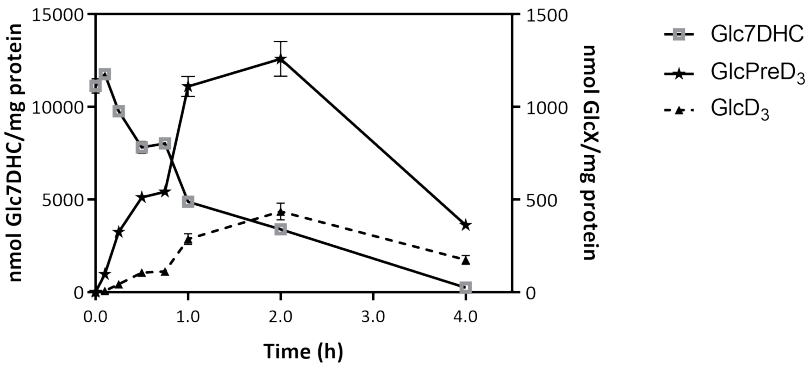


Figure 6. Conversion of Glc7DHC into $GlcPreD_3$ and $GlcD_3$. *Glc7DHC was irradiated with UVB for varied time. Samples were analysed by LC-MS/MS for the presence of Glc7DHC, $GlcD_3$ and the assumed $GlcPreD_3$.*

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).

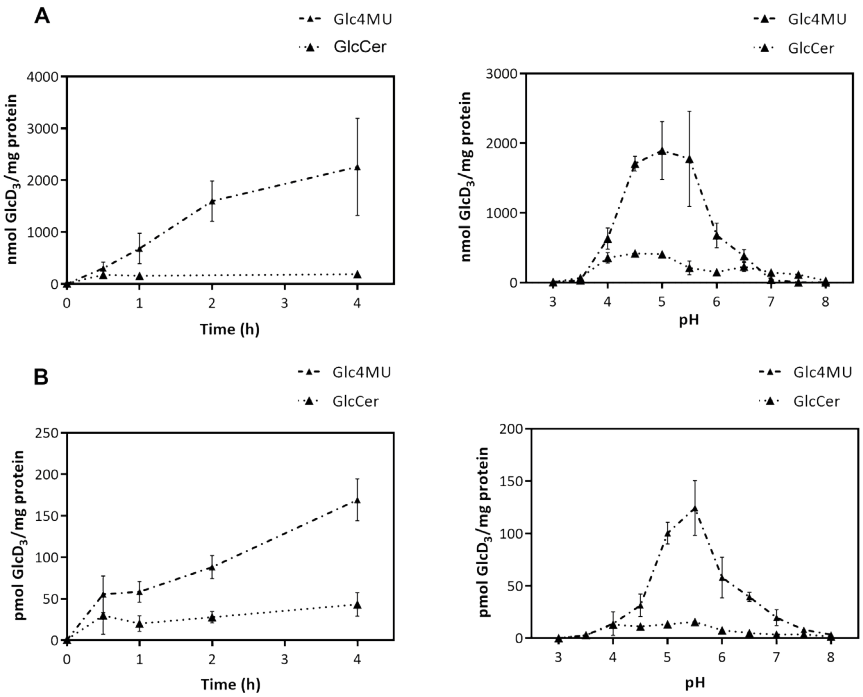


Figure 7. In vitro formation of $GlcD_3$. *A) rGBA was incubated for varied times and varied pH with cholecalciferol (D_3) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer) donor. Formation of $GlcD_3$ was measured by LC-MS/MS (nmol/mg protein). B) Lysates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

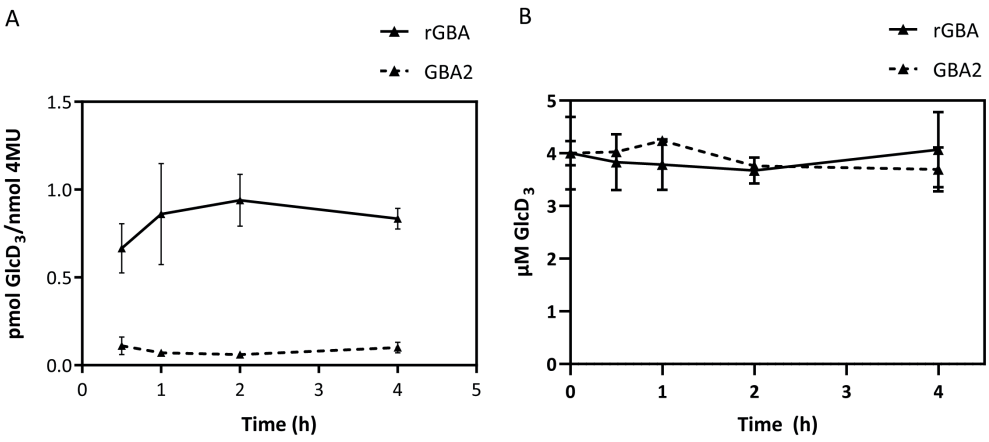


Figure 8. Ratio Transglucosylation and hydrolase activities and degradation of $GlcD_3$. *A) rGBA was incubated for varied times with cholecalciferol (D_3) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc). 4MU-emmitted fluorescence (nmol 4MU/mg protein) was measured a LS-55 Fluorescence 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.

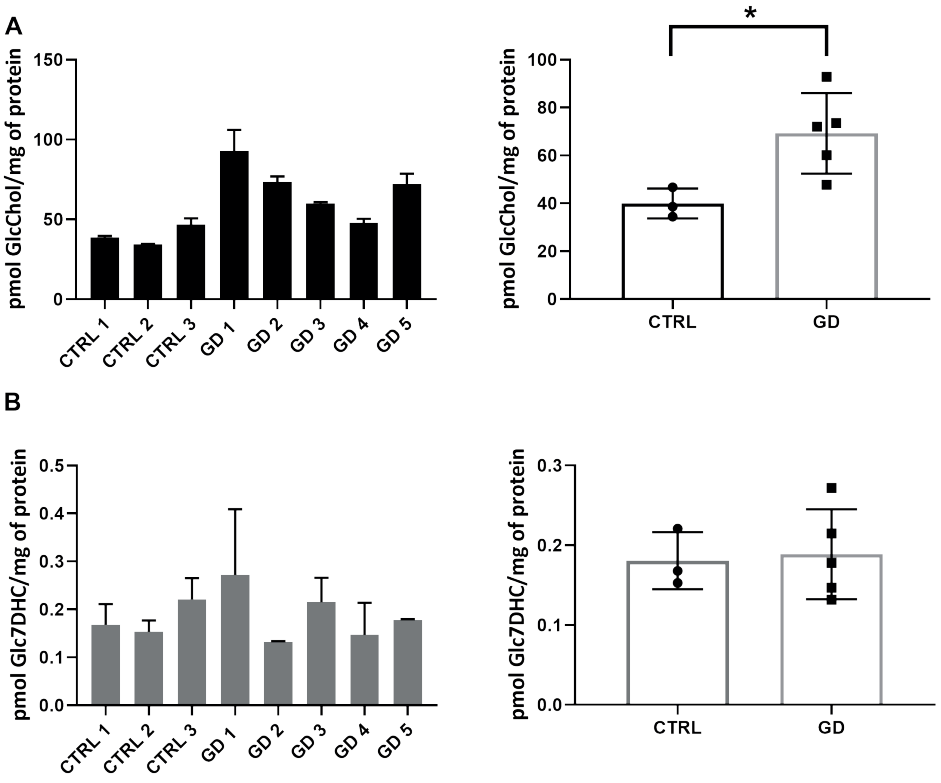


Figure 9. Levels of GlcChol and Glc7DHC in GD spleen. *LC-MS/MS analysis of GlcChol and Glc7DHC occurrence in human control spleens and human GD spleens. Errors bars are standard deviation of technical duplicates. Data were analysed by unpaired Student's t-test. P values <0.05 were considered significant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

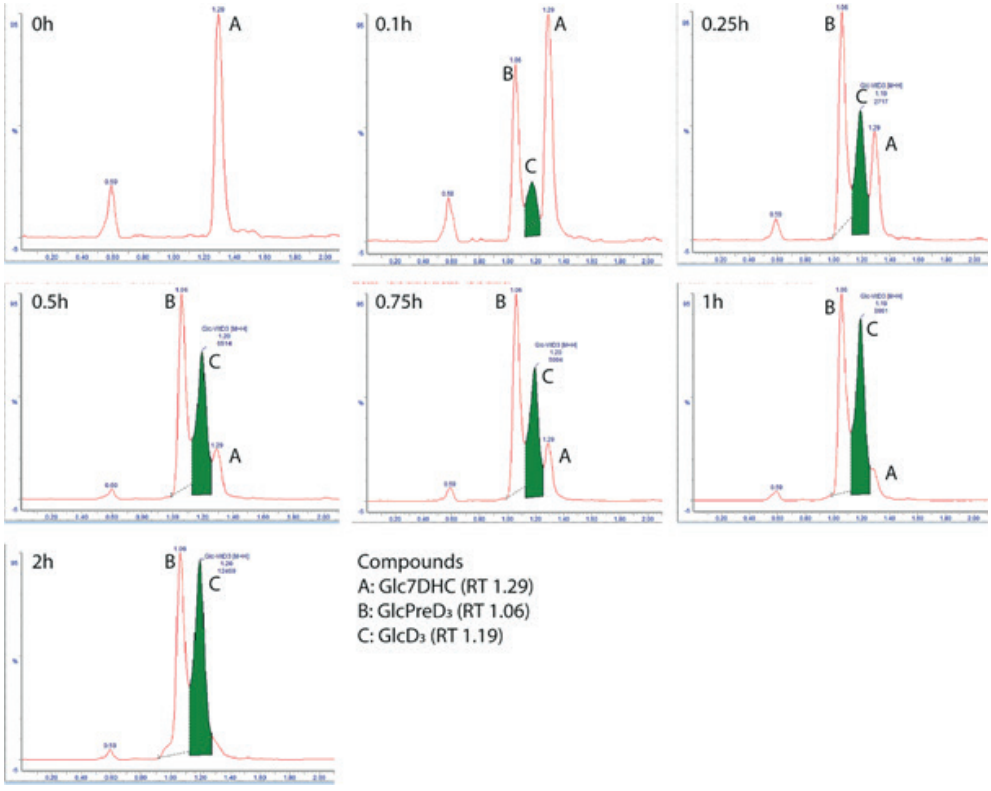
A final consideration is the partial overlap in symptoms between diseases such as SLOS, GD and NPC (Supplemental Table 1). It is appealing to comparatively document for these disorders the presence of glucosylated compounds such as GlcChol, Glc7DHC and $GlcD_3$. Osteoporosis and vitamin D_3 abnormalities 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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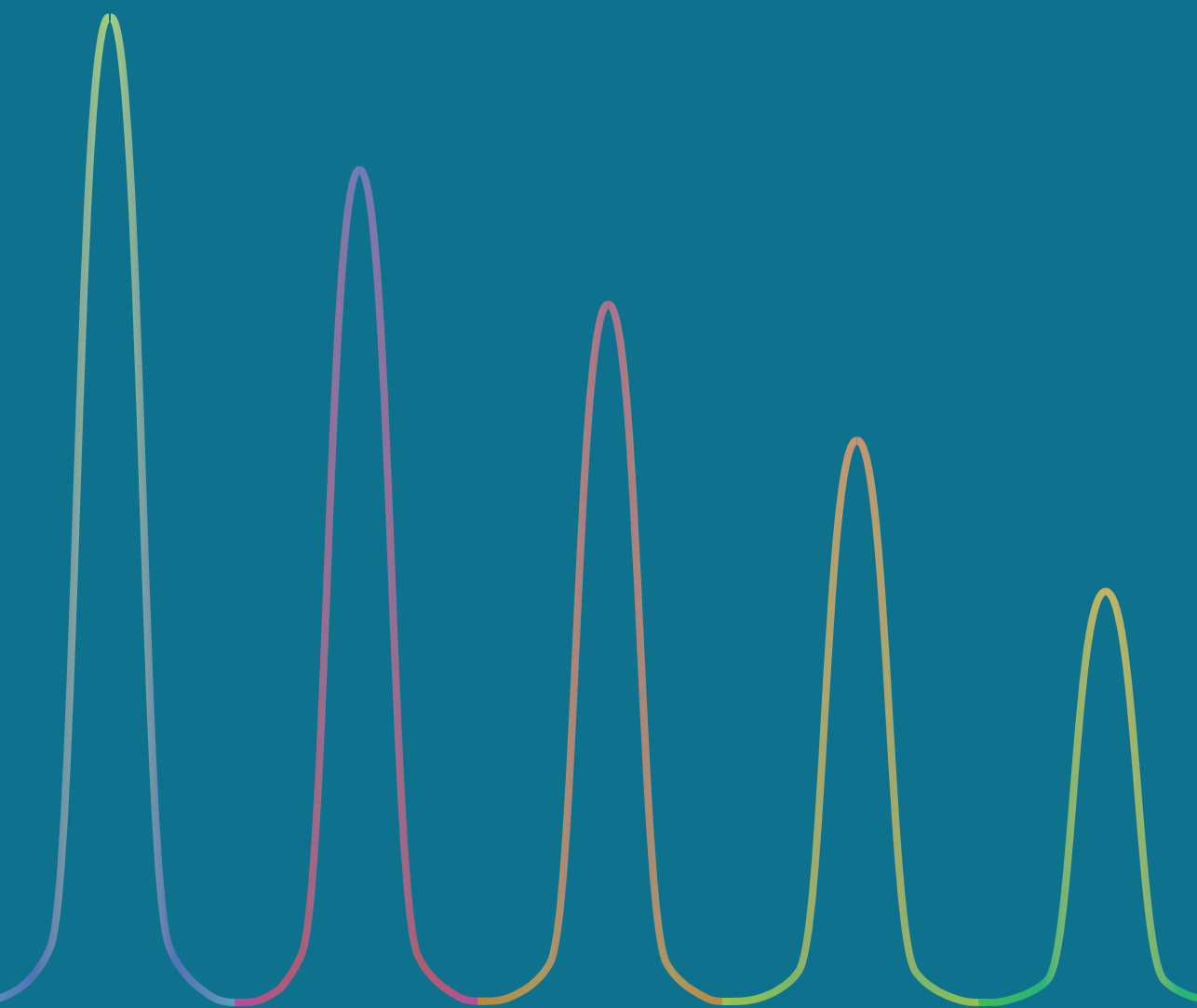

Chapter 5

Potential role of GBA3 in formation and degradation of glycosylated metabolites

To be incorporated in manuscript in preparation

Chapter 5 - Potential role of GBA3 in formation and degradation of glycosylated metabolites

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To be incorporated in manuscript in preparation

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
M.A. : co-supervision
J.M.A. : supervision

Abstract

The recently recognized transglucosylation ability of by the cellular β-glucosidases, lysosomal glucocerebrosidase (GBA) and membrane-associated nonlysosomal glucosylceramidase GBA2, have prompted studies on this for the broad-specific β-glucosidase (GBA3). We studied the capacity of GBA3 *in vitro* to hydrolyze β-glucosides as well as its ability to transglucosylate acceptors. We investigated, with our sensitive quantitative LC-MS/MS method, the *in vitro* formation of GlcChol by GBA3 and the degradation of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. During our study we observed that GBA3 was not able to form GlcChol, nor that GBA3 was able to degrade any of the studied metabolites. The negative findings warrant use of larger amounts of a purified recombinant GBA3.
It is however conceivable that GBA3 is indeed unable to form and degrade GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. The obtained results stimulate further research on GBA3 to synthesize and degrade galactosylated or xylosylated cholesterol, 7-dehydrocholesterol, D_3 (cholecalciferol) or desmosterol.

Introduction

The broad-specific β-glucosidase (GBA3), also known as klotho-related protein (KLrP), is known to be cytosolic and present in kidney, liver, spleen, intestine and lymphocytes of mammals. It is thought to be involved in hydrolysis of xenobiotic glycosides [1, 2]. The GH1 enzyme is thought to be most likely involved in detoxification of glucosylated xenobiotics. Via drinking water, food, medical treatment or pollution these substances are absorbed into the body and require detoxication [3]. Plants are known to contain various α- and β-glucosidases, galactosidases and xylosidases [4]. As we eat plants, these glucosylated metabolites enter our body as glucosylated xenobiotics. GBA3, as broad-specific glycosidase, hydrolyzes substrates with a variety of an α-arabinose, β-glucose, β-galactose or β-xylose moiety linked to a hydrophobic group [5]. Artificial substrates such as 4-methylumbelliferyl-β-D-glucoside and C_6-NBD-glucosylceramide are hydrolyzed by GBA3, but naturally occurring lipids like glucosylceramide (GlcCer) and glucosylspingosine seem poor substrates [1, 2]. GBA3 has been studied in relation to Gaucher disease (GD). The underlying cause of GD is deficiency of glucocerebrosidase (GBA), the enzyme responsible for the hydrolysis of GlcCer into glucose and ceramide. Deficiency of GBA causes lysosomal storage of GlcCer in tissue macrophages, resulting in a broad spectrum of clinical presentations [6-10]. GBA3 is thought not to have any involvement in the clinical manifestation of non-neurological (type 1) GD [1].

The 469 amino acid GBA3 is encoded by the GBA3 gene on the 4p15,31 locus [11-13]. The crystal structure reveals the presence of a characteristic TIM barrel of retaining glycosidase, potentially capable of transglycosylation [14, 15]. GBA3 uses a similar double displacement mechanism as the enzymes GBA and GBA2 capable of transglucosylation [2, 16]. GBA3 has two catalytic glutamate residues in its active site (Glu 165 and Glu 373) [2]. The protonated aglycone departs after nucleophilic attack of the glutamate residue leading to an α-linked covalent glycosyl-enzyme intermediate. The crystal structure of the covalent glycosyl intermediate was elucidated and the double displacement hydrolysis mechanism of GBA3 was confirmed [15].

We examined the ability of GBA3 to perform transglucosylation as well as its ability to glucosylated metabolites that were earlier detected in human tissue.

Results

Formation and degradation of glucosylated metabolites by GBA3

Previous investigations did not point to transglycosylation activity of GBA3 [17]. This was confirmed by the analysis of GlcChol formation via transglycosylation by GBA3 (Figure 1). For this, homogenates of cells overexpressing GBA3 were incubated with cholesterol (Chol) as acceptor and either 4MUGlc or GlcCer as sugar donor. The activity of GBA and GBA2 in the homogenates was included or excluded by adding the following inhibitors: final concentration of 1 mM Conduritol B Epoxide (CBE, GBA1 specific inhibitor) and/or *N*-adamantine-methyloxypentyl-deoxynojirimycin 1 µM AMP-DNM (preferential GBA2 inhibitor). The sugar donor, either GlcCer (0.1 mM) or 4MUGlc (3.7 mM), was combined with 0.1 mM of Chol. The reaction was incubated for 1 hour at 37°C in 100 mM HEPES buffer (pH 7.0). The formation of GlcChol was monitored by LC-MS/MS.

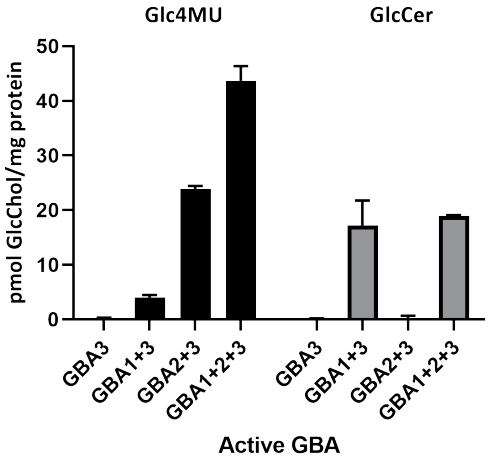

Figure 1. In vitro formation of GlcChol. *Lysates of cells overexpressing GBA3 were incubated for varied times with cholesterol (Chol) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer) donor. Formation of GlcChol was measured by LC-MS/MS (pmol/mg protein). GBA and GBA2 was included or excluded by making use of inhibitors, 1 mM Conduritol B Epoxide (CBE, GBA1 specific inhibitor) and/or 1 µM AMP-dNM (GBA2 specific inhibitor).*

We next investigated the ability of GBA3 to degrade GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. These metabolites were synthesized at the Department Bi-organic Synthesis. Each of the compounds was incubated at 4 µM at 37°C for varied times with homogenates of cells overexpressing GBA3. To exclude action of GBA and GBA2 the homogenates were pretreated with the irreversible GBA inhibitor ME656 or the preferential GBA2 inhibitor AMP-DNM. The reduction of the metabolites during a 4 hour incubation was monitored by LC-MS/MS. No degradation of the glucosylated metabolites observed (Figure 2).

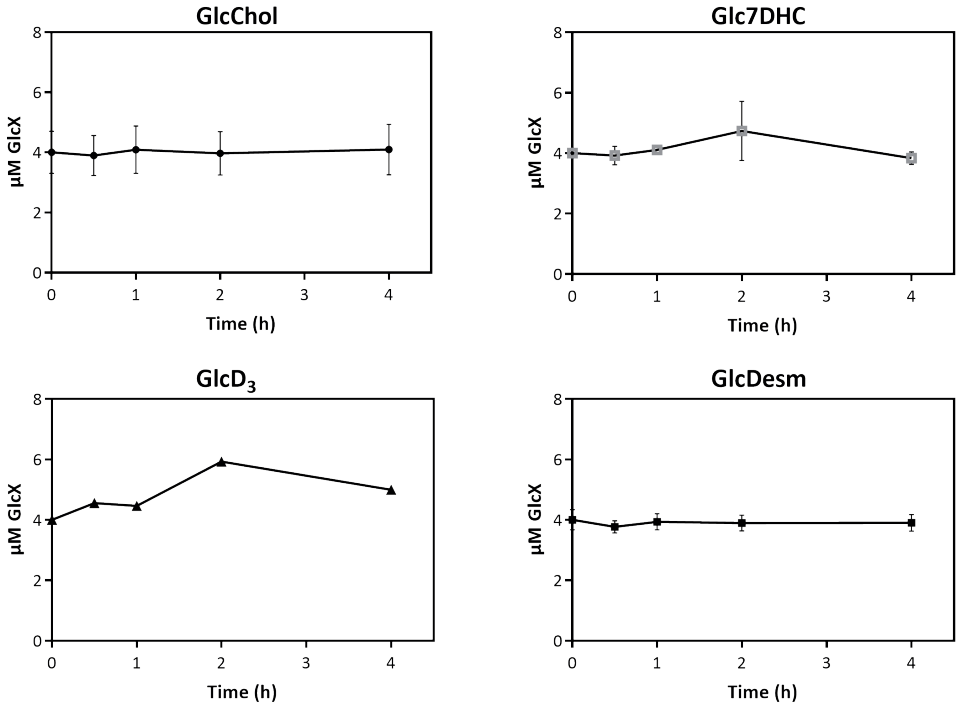

Figure 2. Degradation by GBA3. *GBA3 was incubated for varied times with 4 µM GlcChol, 4 µM Glc7DHC, 4 µM $GlcD_3$ or 4 µM GlcDesm. Degradation of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm was measured by LC-MS/MS (µM) and corrected for amount of protein. GBA and GBA2 activity was excluded by using the inhibitors: 50 nM ME656 (GBA1 specific inhibitor) and/or 1 µM AMP-dNM (preferential GBA2 inhibitor).*

Discussion

The obtained data indicate that GBA3 is not able to perform transglucosylation of cholesterol to generate GlcChol. Moreover, GBA3 seems not able to significantly degrade the glucosylated metabolites GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. Previously presented data shows that the latter compounds are all endogenous. Lack of metabolism of GBA3 might be contributing to this, as GBA3 is known to be active towards glycosylated metabolites with a rigid, planar, aglycon, such as quercetin [15, 18]. The aglycon specificity of hCBG has mainly been studied for xenobiotic β-D-glycosides [19]. β-D-glucosides of isoflavones, flavonols and flavones were identified as good substrates. A flat aromatic molecule is a favorable substrate compared to aliphatic analogues. Glucosides of dihydrochalcones (phlorizin) and anthocyanins were not substrates [18]. GBA3 hydrolyzes cyanogenic glucosides and phenolic glucosides [19]. Modelling and crystallography with recombinant GBA3 might confirm the observed lack of metabolism of GBA3 for GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm. Quercetin-glucoside is in this case good positive control.

In addition, investigations of the activity of GBA3 towards galactosylated or xylosylated forms of metabolites is of interest. In plants xylose is a major sugar and several plant β-xylosides compounds are known [20]. Via food uptake, some of these might enter the body and might act as xylose-donors for transxylosylation reactions [21, 22].

Most importantly, larger scale production and purification of GBA3 seems warranted to definitively exclude (or establish) a role of the enzyme in the metabolism of glycosylated metabolites in humans. Such role would be relevant in view of the frequent inherited defect in GBA3 [1].

Acknowledgements

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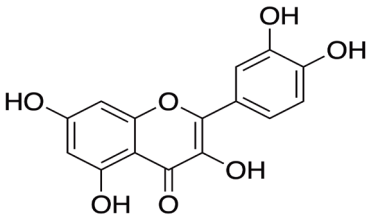
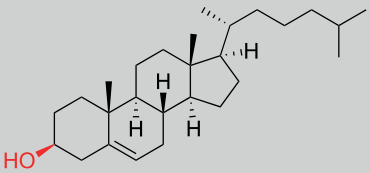
Table 1. Chemical structures of rigid planar hydrophobic compounds. *Presented are compounds Quercetin, of which the glucosylated compounds fits in the pocket of GBA3 [15], cholesterol (chol), 7-dehydrocholesterol (7DHC), Desmosterol (Desm), vitamin D (cholecalciferol, D_3) of which the glycosylated compounds do most probably not fit the pocket of GBA3.*

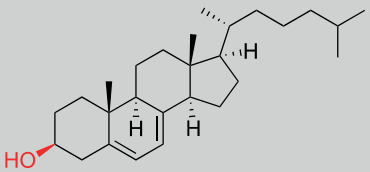
Quercetin



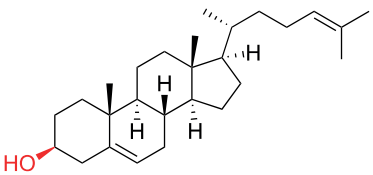
Chol



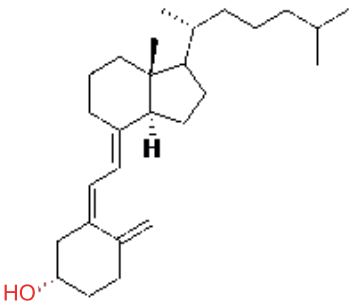
7DHC



Desm



D_3



Experimental procedures

Materials

As in previous work the following pure grade chemicals were used: cholesterol (Sigma-Aldrich, St Louis, MO, USA), D-glucosyl-β-1,1'*N*-oleoyl-D-*erythro*-sphingosine (C18:1-GlcCer) (Avanti Polar Lipids, Alabaster, AL, USA), 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc) (Glycosynth™, Winwick Quay Warrington, Cheshire, England). The department of Bio-organic Synthesis (University of Leiden, The Netherlands) synthesized the used inhibitors following previously described procedures: GBA1 inhibitor ME656 [23], Conduritol B Epoxide (CBE, GBA1 specific inhibitor) [24] and AMP-DNM (GBA2 specific inhibitor) [25]. As specific lipid substrates were used: $^{13}C_6$-β-GlcChol [24], GlcChol, $^{13}C_6$-β-Glc7DHC, Glc7DHC and $GlcD_3$, $^{13}C_6$-β-GlcDesm and GlcDesm synthesized at the Bio-organic Synthesis department (University of Leiden, The Netherlands).

As source of GBA3, lysates of HEK293T-cells overexpressing the enzyme were used.

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

Methods

In vitro assay of transglucosylation activity. Lysates of HEK293T cells overexpressing GBA3 were used to determine transglucosylation activity of the enzyme GBA3. Specific inhibitors were used to inhibit the enzymatic activity of GBA and GBA2 in the lysates. The assays were performed as described earlier [17] with the following modifications. The samples contained 40 µL homogenate of GBA3 overexpressing cells, to which was added a final concentration of 1 mM Conduritol B Epoxide (CBE, GBA1 specific inhibitor) and/or 1 µM AMP-DNM (GBA2 specific inhibitor) or 100 mM HEPES buffer (pH 7.0). The glucose donor was either 4-methylumbelliferyl-β-D-glucoside (4MUGlc) or glucosylceramide (GlcCer). The final concentrations in the reaction were 100 µM C18:1-GlcCer or 2.8 mM 4MUGlc. As final concentration of acceptor 100 µM of Chol was added to the reaction.

The transglucosylation reaction was carried out in the presence of additional 200 µL 100 mM HEPES buffer (pH 7.0) and 12.5 µL of ethanol. After 1h incubation at 37°C, the transglucoylsation reaction was terminated by putting samples on ice, the samples were subjected to lipid extraction according to Bligh and Dyer [26] by addition of methanol, chloroform and water (final volumes: 1:1:0.9, v/v/v). As internal standard 50 µL aliquots of 0.1 pmol/µL $^{13}C_6$-β-GlcChol each in methanol were added. 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/MS.

Degradation of GlcChol, Glc7DHC and $GlcD_3$ and GlcDesm. The ability of homogenates of HEK293T cells overexpressing GBA3 to degrade GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm was monitored over time. Therefore 4 µM of GlcChol, Glc7DHC, $GlcD_3$ or GlcDesm was added to 40 µL of homogenate of GBA3 overexpressing cells, to which was added 10 µL of 50 nM ME656 (GBA1 specific inhibitor) and 1 µL of 1 µM of AMP-DNM or 100 mM HEPES buffer (pH 7.0). The reaction was carried out in the presence of additional 5 µL of ethanol. All samples were prepared for LC-MS/MS as described previously and measured by LC-MS/MS for GlcChol, Glc7DHC, $GlcD_3$ or GlcDesm levels.

Protein determination. Performed with The Pierce BCA Protein Assay kit (Thermo Scientific) and absorbance was measured at 562 nm in EL808 Ultra Microplate Reader (BIO-TEK Instruments Inc.).

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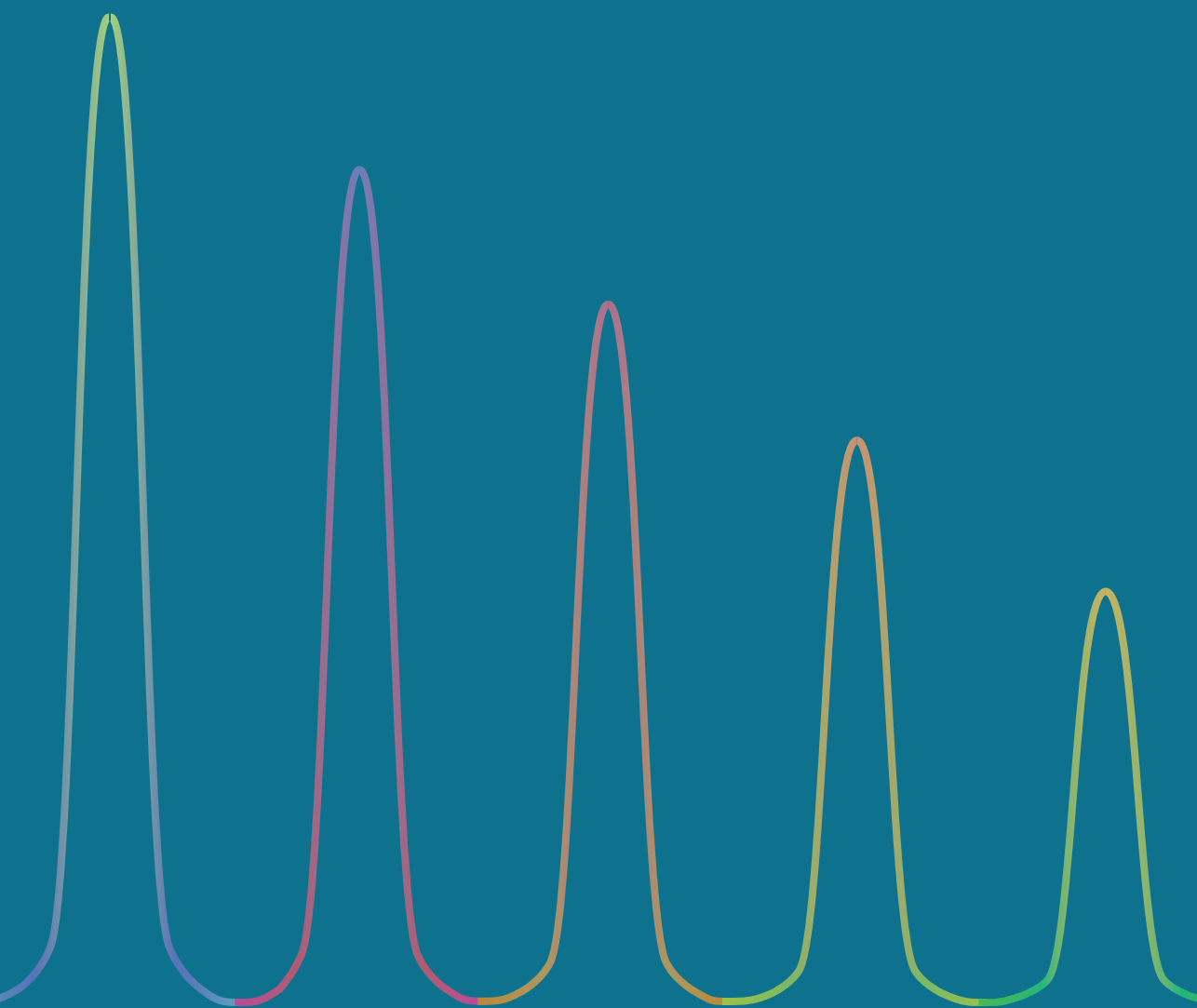

Chapter 6

General discussion and perspectives for future research

General discussion

The topic of this thesis is the discovery and characterization of new glucosylated metabolites and their pathophysiological consequences. The lysosomal enzyme glucocerebrosidase (GBA) is a retaining β-glucosidase that cleaves glucose from the glycosphingolipid glucosylceramide (GlcCer) employing the double displacement mechanism [1]. During regular hydrolysis of GlcCer by GBA, the glucose moiety of the substrate becomes linked to the nucleophile E340 of the enzyme and is next released to water as acceptor [1, 2]. It was recognized with *in vitro* experiments that alternatively, via a so-called transglucosylation reaction, GBA transfers the bound glucose moiety onto cholesterol (Chol) to form glucosyl-β-cholesterol (GlcChol) [3-5]. Likewise, the cytosol-facing retaining β-glucosidase GBA2 is able to perform transglucosylation, again generating from the donor GlcCer the product GlcChol [3]. It has become subsequently clear that normally GBA degrades GlcChol and GBA2 generates the glucosylated sterol [3]. However, in Niemann Pick disease type C (NPC), where cholesterol accumulates in lysosomes due to an inherited defect in its efflux, even GBA generates GlcChol via transglucosylation [3]. Natural occurrence of GlcChol has been demonstrated in several cells and tissues, but its physiological role still remains to be elucidated. Yet, the observed formation of GlcChol via transglucosylation raises the intriguing question whether other metabolites besides cholesterol might comparably act as acceptors in transglucosylation. Key candidates in this respect are compounds similar in structure to Chol.

One of the major aims of this thesis work was to develop a sensitive LC-MS/MS method to quantify newly discovered glycosylated metabolites, like glucosylated 7-dehydrocholesterol (Glc7DHC), glucosylated vitamin D_3 ($GlcD_3$) and glucosylated desmosterol (GlcDesm) in human body fluids and tissue. This goal was reached by further development of the LC-MS/MS method for quantitative measurement of GlcChol [3] (**Chapter 2** of this thesis). With the desired method in place we could demonstrate the *in vitro* formation and degradation of GlcDesm (**Chapter 3** of this thesis) and that of Glc7DHC and $GlcD_3$ (**Chapter 4** of this thesis). Furthermore, we demonstrated the presence of Glc7DHC and GlcDesm in biological samples (**Chapter 2**, **Chapter 3** and **Chapter 4** of this thesis). In **Chapter 5** of this thesis the transglycosylation potential of the broad-specific β-glucosidase (GBA3) is discussed. Transglucosylation by GBA3 has not yet been detected and its ability to perform transxylosylation warrants further investigation. Xylosylated cholesterol occurs [6], however xylosylation of 7-dehydrocholesterol is unclear (see later in this discussion).

The research described in this thesis excites directions for further investigations that are discussed below.

Perspectives for future research

New metabolites to be explored

The first direction of new research to be discussed is the search for additional metabolites, related in structure to cholesterol, that are transglucosylated as well. Besides being structurally related to 7DHC and Desm, cholesterol is a precursor for other metabolites such as oxysterols, bile acids, and steroid hormones [7]. As the chemical structure of these downstream metabolites resembles their precursors, they are conceivably also subject to transglucosylation. The three different classes of metabolites are separately discussed below.

A. Oxysterols.

Oxysterols are associated to several diseases, like atherosclerosis, AD, multiple sclerosis, Huntington's disease, Niemann Pick type C disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) [8], Smith-Lemli-Opitz Syndrome (SLOS, a disease with dysfunctional DHCR7) [8-12], autism spectrum disorders and spastic paraplegia type 5 [8]. Formation of oxysterols from Chol, 7DHC or Desm occurs via members of the cytochrome P450 family, in particular 27-hydroxylase (CYP27A1), 24-hydroxylase (CYP46A1), 7α-hydroxylase (CYP7A1), Cytochrome P450Scc (CYP11A1) or via autoxidation processes (oxidation and epoxidation) [8, 13, 14]. Oxysterols formed by auto-oxidation, like 7-hydroxyperoxycholesterol, 7-ketocholesterol and 7β-hydroxycholesterol, appear during oxidative stress and have been found in association with atherosclerosis, Parkinson's disease, Niemann Pick disease type C and SLOS [8, 13, 14]. Furthermore, they occur in our diet [13]. The enzymatically formed oxysterols activate LXRs [14], but are also important in bile acid and steroid production [8].

In brain and adrenal glands CYP46A1 is responsible for the conversion of cholesterol into the oxysterol 24S-hydroxycholesterol [15-17]. 24S-hydroxycholesterol is important in cholesterol homeostasis in the brain. It crosses the blood-brain barrier and thus allows exit of cholesterol building block from the brain. Via the blood circulation 24S-hydroxycholesterol moves towards the liver for further conversion into bile acids [17, 18]. The enzyme CYP46A1 is also able to convert Desm to two oxysterol products, 24S,25-epoxycholesterol and 27-OH-desmosterol. It can convert 7-DHC into the oxysterols 24-hydroxy-7-dehydrocholesterol (24-(OH)7DHC) and 25-hydroxy-7-dehydrocholesterol (25-(OH)7DHC [8, 19].

B. Steroid hormones

Steroid hormones regulate numerous processes in the central nervous system and in peripheral organs. They act through binding with specific nuclear receptors and mediate gene transcription, inducing behavioral and developmental responses and metabolic actions. Steroid hormones are thought to cross the blood-brain barrier by diffusion [20, 21]. Their structure exists of a tetracyclic (cyclopentaphenanthrene) skeleton, for which Chol is the main precursor [21,

22]. Formation of steroid hormones takes place in the adrenal gland, skin, testis and placenta. The enzyme CYP11A1 modifies cholesterol, to pregnenolone, the precursor of progesterone, cortisol and testosterone [8, 23-26]. On the other hand, desmosterol is also converted by CYP11A1 to the pregnenolone derivative 5-pregnene-3β,20β-diol, which has a similar structure to the cholesterol derivative pregnenolone [27, 28]. In the skin CYP11A1 transforms 7DHC into 7-dehydropregnenolone (7DHP), which is a precursor for several steroidal 5,7-dienes and 4,7-dienes, like 7-dehydroprogesterone [29-33]. The 5,7-diene precursors and secosteroidal products have inhibitory effects on cell proliferation and induce differentiation without calcemic activity [34]. In the skin, D_3 is also an substrate for CYP11A1, resulting in the formation of 20-hydroxycholecalciferol ($20(OH)D_3$) [35, 36], which exhibits antiproliferative activity and inhibits human melanoma growth [36, 37].

C. Bile acids

Bile acids are of great importance for intestinal nutrient absorption, biliary secretion, maintenance of metabolic homeostasis by acting as signaling molecules in lipid, glucose and energy metabolism. They are amphipathic molecules with powerful detergent properties, due to their tetracyclic skeleton with hydroxyl and carboxyl groups on the opposite site of the hydrophobic tail. The complete conversion of cholesterol to bile acids involves 17 distinct enzymes. These enzymes modify the steroid ring and the side chain of cholesterol. The biosynthetic pathway consists of two paths, the neutral bile acid pathway (classic pathway) initiated by the enzyme 7α-hydroxylase (CYP7A1) and the acidic pathway, initiated by 27-hydroxylase (CYP27A1) [15, 38]. CYP7A1 is present in tissues like liver, macrophages, retina and brain nerve cells. Inside the liver the main production of bile acids occurs (200 to 600 mg bile acid per day). CYP7A1 catalyzes the conversion of cholesterol into the oxysterol 7α-hydroxycholesterol [15, 39, 40]. 7α-Hydroxycholesterol is a precursor for the primary bile acids cholic acid and chenodeoxycholic acid. As a negative feedback loop, CYP7A1 activity is inhibited by the oxysterol 7-ketocholesterol, which is produced by CYP7A1 itself via 7DHC oxidation to 7-ketocholesterol or via radical attack by reactive oxidative species (ROS) on cholesterol [41, 42]. Furthermore, CYP7A1 converts 7DHC into 7α,8α-epoxycholesterol [8, 41, 43], a metabolite specifically present in plasma of SLOS patients [43]. CYP27A1, initiating the acidic pathway, is present in liver and lungs, and converts cholesterol to 27-hydroxycholesterol and other oxysterols like cholestenoic acid [15, 38]. Mice with a homozygous knockout, show hepatomegaly, increased cholesterol synthesis and affected fatty acid and bile acid synthesis [38]. In humans the deficiency of CYP27A1 leads to cerebrotendinous xanthomatosis (CTX)[44]. A disease associated with accumulation of cholesterol in the Central Nerves System (CNS) [45], excretion of bile alcohols and low fecal bile acid excretion [38, 46]. Some cases even develop hepatomegaly [47], as observed in CTX mice [38]. Furthermore, CTX is associated with osteoporosis and high chance on bone fractures [48, 49]. This might relate to 7DHC and D_3, both being

a substrate for CYP27A1. In SLOS patients 7DHC levels are elevated, as well as levels of 25(OH)D_3 [51]. SLOS patients do not show association with osteoporosis, indicating that elevation of 7DHC and 25(OH)D_3 protects against osteoporosis [8, 43, 50] In CTX patients, 7DHC levels are also elevated [52], while conversion of D_3 into 25(OH)D_3 by CYP27A1 is impaired[53], resulting in lower levels of 25(OH)D_3 [54]. This might explain why SLOS patient do not develop osteoporosis, while CTX patients do.

Within the three discussed classes of metabolites above transglycosylation of some compounds might occur. After selection of the most likely candidates, comparable research as performed in this thesis (**Chapter 2**, **Chapter 3** and **Chapter 4**) should be considered.

Glucosylated metabolites: the missing link?

The biological function and pathophysiological relevance of glucosylated metabolites in the body is a complete new field of research. In this paragraph it is discussed which kind of impact those metabolites might have, based on literature on the non-glycosylated metabolites.

Gaucher disease (GD) is due to inherited defects in GBA, the lysosomal glucocerebrosidase responsible for breakdown of the glycosphingolipid glucosylceramide (GlcCer) [55-57]. Consequently, GlcCer metabolism is disturbed. Gaucher disease has may manifest with various symptoms ranging from hypersplenism, skeletal complications, hepatomegaly and neurodegeneration [56, 58-61]. Interestingly patients with the same mutation develop different phenotypes, as even twins with the same genetic background show heterogeneous phenotypes [62]. A phenomenon which is also observed in CTX patients [46] and Desmosterolosis, in which the same mutation leads to different phenotypes [63]. Some of the noted symptoms in Gaucher disease patients might point to disturbances in the pathways of desmosterol, 7DHC and cholesterol metabolism. If these pathways are indeed involved than the differences between twins might be related to their food intake, as desmosterol, 7DHC and cholesterol are compounds that are present in our food [14, 64-69]. The discovery of GlcChol is of great interest. We suspect that, as GlcChol levels are increased in plasma of Gaucher patients [3], glucosylated 7DHC and Desm might be present in Gaucher patients as well. GD is not the only disease in which GlcChol was discovered, also in NPC patients elevated levels of GlcChol were detected [3].

The discovery of GlcChol in both of these diseases makes us wonder whether in other disease, like SLOS with high 7DHC levels and Desmosterolosis (defect in DHCR24, resulting in high levels of Desm) [70, 71], also metabolites as Glc7DHC and GlcDesm might be present. Other diseases warranting such investigations are vitamin-D-dependent Rickets, CTX and Lathosterolosis (defect in SC5D, with decreased levels of 7DHC) [72, 73]. All these diseases might have glucosylated metabolites playing a role in the pathology of the disease. An overview of the pathways of Desm, 7DHC and Chol is presented in Figure 1.

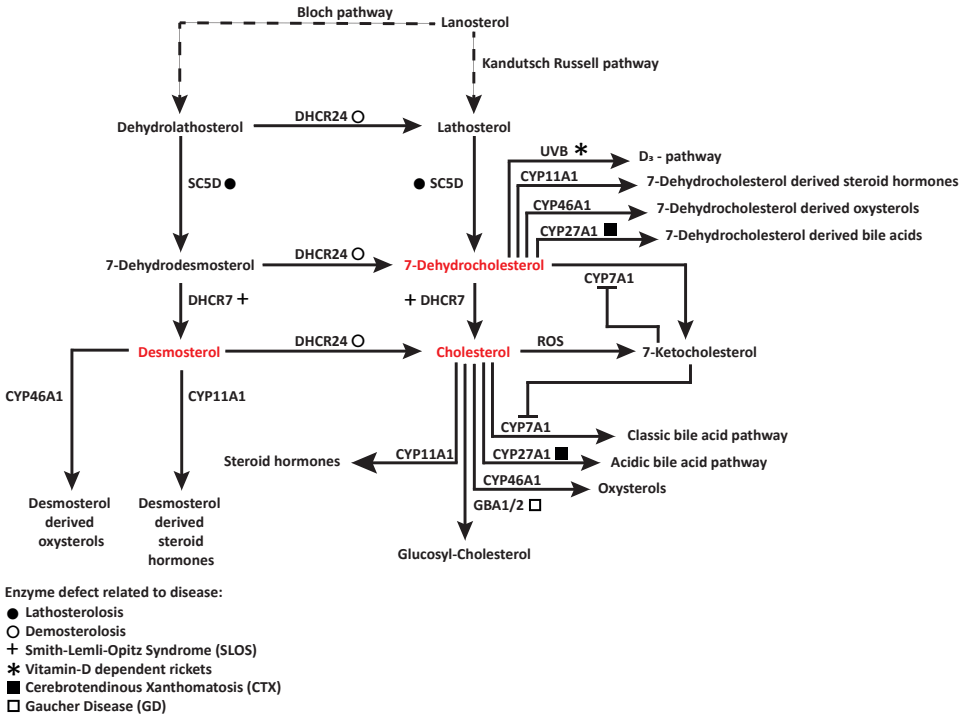

Figure 1. Pathways of desmosterol, 7-dehydrocholesterol and cholesterol related to disease.
Displaying the pathways of desmosterol, 7-dehydrocholesterol and cholesterol. Involved enzymes are Sterol C5-desaturase (SC5D), 7-Dehydrocholesterol Reductase (DHCR7), 24-Dehydrocholesterol Reductase (DHCR24), 7α-hydroxylase (CYP7A1), Cytochrome P450Scc (CYP11A1), 27-hydroxylase (CYP27A1), 24-hydroxylase (CYP46A1), glucocerebrosidase 1 and 2 (GBA1/2). Defective enzymes are marked and related to the corresponding diseases, lathosterolosis, desmosterolosis, Smith-Lemli-Opitz Syndrome (SLOS). Vitamin-D dependent rickets, Cerebrotendinous Xanthomatosis (CTX) and Gaucher Disease (GD).

Potential acceptor abnormality that might promote causing glucosylated metabolites abnormalities

Table 1a summarizes literature on key metabolites of the 7DHC/D_3 and cholesterol pathways for various relevant diseases including Niemann Pick type C. In NPC elevated levels of both GlcCer and Chol are reported [3]. Earlier research showed that in plasma of NPC patients elevated levels of GlcChol occur as compared to healthy individuals. Table 1b shows a hypothetical prediction of glucosylated metabolite in various relevant diseases.

Table 1a. Metabolites in disease. *Levels of lathosterol (Latho), cholesterol (Chol), 7-dehydrocholesterol (7DHC), desmosterol (Desm), vitamin D_3 (D_3), 25-hydroxyvitamin D_3 (25(OH)D_3), 1α-25-dihydroxyvitamin D_3 (1,25(OH)2D_3) for each of the diseases Lathosterolosis, SLOS, Desmosterolosis, Cerebrotendinous Xanthomatosis (CTX), Vitamin D dependent Rickets and Gaucher Disease Type I, II and III and Niemann Pick Disease Type A, C1 and C2. Elevated indicated by +, decreased indicated by -, normal levels indicated by N. * Levels of D_3 are not measured within Desmosterolosis, but patients show osteosclerosis, which is related to high levels of D_3. Therefore levels are expected to be either elevated or normal. ** As levels of D_3 are unmentioned for Vitamin D- dependent rickets patients, we suspect based on that the enzyme defect is downstream of D_3 formation that levels are either elevated or normal. *** Levels of Desm are only mentioned for Type C1, due to the large overlap between Type C1 and C2 it is suspected that levels of Desm in Type C2 are also elevated as in Type C1.*

Disease		Enzyme Defect	Latho	Chol	7DHC	Desm	D_3	25(OH)D_3	1,25(OH)$_2D_3$
	Lathosterolosis [72-77]	SC5D	+	-/N	-/N	-	-		
	SLOS [72, 78, 79]	DHCR7		-	+	-	+		
	Desmosterolosis [63, 70, 80-86]	DHCR24		-/N	+/N	+	+/N*		
	CTX [44-46, 48, 49, 52, 54, 87-89]	CYP27A1	+	+/N	+		+/N	-	N
Vitamin D - dependent Rickets	*Type 1A [90-92]*	CYP27B1					+/N**	+/N	-
	Type 1B [90-93]	CYP2R1					+/N**	-	N
	Type 2A [90-92, 94-96]	VDR					+/N**	+/N	+
Gaucher Disease	*Type I [3, 97-103]*	GBA		-				-/N	
	Type II [101]	GBA		-					
	Type III [101, 104]	GBA		-					
Niemann Pick Diseases	*Type A [105-107]*	SMPD1		+					
	Type C1 [3, 108-110]	NPC1		+		+			
	Type C2 [3, 111-113]	NPC2		+		+***			

In NPC elevated levels of both GlcCer and Chol are reported. Earlier research showed that in plasma of NPC patients elevated levels of GlcChol occurs as compared to healthy individuals. Table 1b shows a hypothetical prediction of glucosylated metabolites.

Table1b. Hypothetical prediction of Glucosylated compounds in disease. *Shows the predicted levels of the glucosylated compounds GlcChol, Glc7DHC, GlcDesm and $GlcD_3$. Known levels of are indicated in bold. Elevated indicated by +, decreased indicated by -, normal levels indicated by N, undetected is indicated by a U, unpredicted is indicated by a blank spot.*

	Disease	GlcChol	Glc7DHC	GlcDesm	$GlcD_3$
	Lathosterolosis *[72-77]*	-/N	-/N	-	-
	SLOS *[72, 78, 79]*	-	+	-	+
	Desmosterolosis *[63, 70, 80-86]*	-/N	+/N	+	+/N
	CTX *[44-46, 48, 49, 52, 54, 87-89]*	+/N	+		+/N
Vitamin D - dependent Rickets	*Type 1A* *[90-92]*				+/N
	Type 1B *[90-93]*				+/N
	Type 2A *[90-92, 94-96]*				+/N
Gaucher Disease	*Type I* *[3, 97-103]*	**+**	**N**	**+**	**U**
	Type II *[101]*				
	Type III *[101, 104]*				
Niemann Pick Diseases	*Type A* *[105-107]*	+			
	Type C1 *[3, 108-110]*	**+**	+	+	+
	Type C2 *[3, 111-113]*	**+**	+	+	+

Untargeted discovery of glycosylated metabolites

The human body was known to contain glucosylated metabolites, ranging from the simplest glycosphingolipid GlcCer [114] to GlcChol [3, 4]. This thesis demonstrates that also Glc7DHC, $GlcD_3$ and GlcDesm occur. More unknown glucosylated metabolites might be present within the human body. Hydrophobic alcohol acceptors X (X = R-OH) might be suitable for formation of a R-β-glucoside. To detect these a so-called 'Transbody' substrate was collaboratively designed and synthesized with the Bio-organic Synthesis department, Leiden Institute of Chemistry. This 'Transbody' is a modified glucose donor to be used in reverse metabolomics (Figure 2).

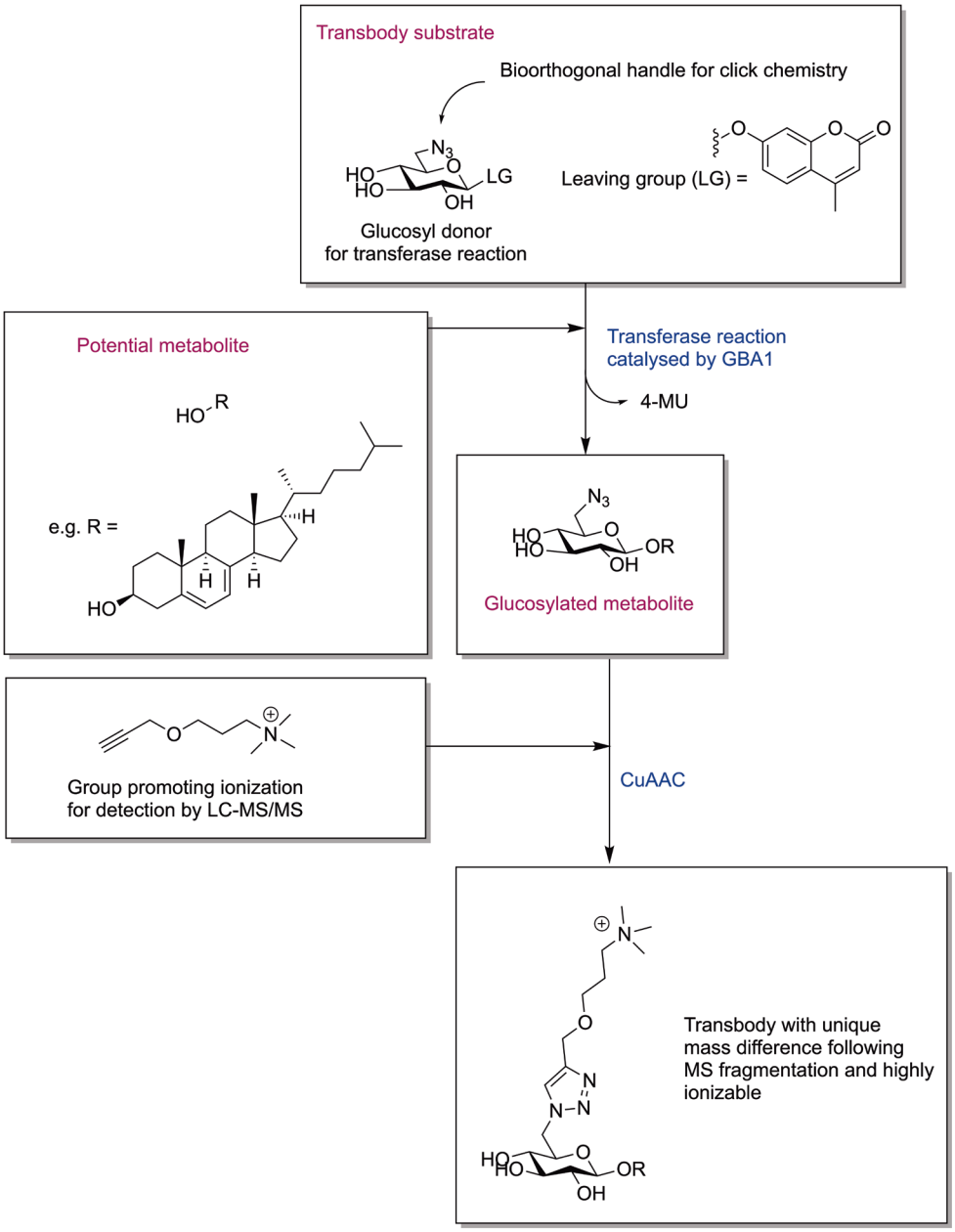

Figure 2. Reverse metabolomics workflow for the discovery of glucose-modified metabolites.

As ‘Transbody’ substrate a C_6-azide-4MUGlc was synthesized. Incubation with recombinant GBA and selected tissue lysates or selected pure alcohols, are expected to result in novel C_6-azide-GlcX. These should be detectable with a specifically developed LC-MS/MS method. For this purpose, the azide should be converted to a group promoting ionization and detection by formation of a unique mass difference following MS fragmentation. Trial experiments were conducted, in order to test the principle. We were able to setup a specific LC-MS/MS method for the detection of C_6-azido-GlcChol. With this method we tested the ability of GBA and GBA2 to transglucosylated the given ‘Transbody’ substrate, C_6-azide-4MUGlc, into C_6-azido-GlcChol. Unfortunately, the ability of GBA to transglucosylate was four times less for the ‘Transbody’ than for the normal 4MUGlc substrate and GBA2 was not able to transglucosylate the ‘Transbody’. An obstacle was the click-chemistry needed to convert the azide group into the ionization and detection promoting group. Further optimization is still required. Alternatively, another modification at C_6 of the glucose-donor substrate is designed preventing the need for click chemistry. Studies on this are presently undertaken at the departments of Medical Biochemistry and Bio-organic Synthesis.

The development of the ‘Transbody’ gives the opportunity to detect known and unknown glucosylated metabolites in biological samples. In **Chapter 2** we present a method for measuring the known glucosylated metabolites GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm within biological samples, such as spleen, plasma, breastmilk and skin. As a limited cohort for each biological sample was available, increasing sample numbers are required for further research. This further research includes the ‘Transbody’ to detect unknown glucosylated metabolites.

Formation and occurrence of Xylosylated metabolites

In line with the conclusion of **Chapter 5** of this thesis, we suggested that xylosylated metabolites might be good substrates for GBA3. The lysosomal β-glucosidase, glucocerebrosidase (GBA) has shown the ability to hydrolyze β-glucosidic substrates and transglucosylates sterols such as cholesterol, 7-dehydrocholesterol and desmosterol to the glucosylated forms GlcChol, Glc7DHC and GlcDesm. Besides hydrolyzing β-glucosidic substrates, GBA is also able to use xylose as sugar donor and perform transxylosylation, at least reaction cholesterol. By transxylosylation xylosyl-cholesterol is formed and this metabolite can act as an acceptor for another xylose to form di-xylosyl-cholesterol as well [6]. As cholesterol can be xylosylated, we conducted some trial experiments on xylosylating 7-dehydrocholesterol. In these experiments GBA has shown potential in transxylosylating 7DHC into Xyl7DHC. These findings prompt further exploration of transxylosylation of 7DHC, the role of Xyl7DHC within the body and the ability of GBA3 to degrade xylosylated compounds.

Conclusion

This thesis describes new LC-MS/MS detection and quantification of discovered glucosylated metabolites Glc7DHC, $GlcD_3$ and GlcDesm. Detection of these metabolites was possible within human samples, such as skin, plasma, breastmilk, but also spleen of healthy and diseased patients. The detection of the glucosylated metabolites allows follow-up research in Gaucher disease patients. It will be of interest to establish whether Glc7DHC and GlcDesm are abnormal in diseases such as SLOS and Desmosterolosis. For this patient material, such as plasma, should be investigated. Even in other diseases such as lathosterolosis, CTX, Vitamin D-dependent rickets and Niemann Pick diseases quantification of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm should be considered. They might provide clues for novel biomarkers and therapies. The observed natural occurrence of the described glucosylated metabolites prompt a search for additional glucosylated metabolites, within the groups of cholesterol-look-a-like structures, such as oxysterols, steroid hormones and bile acids. New approaches, as the described reversed metabolomics approach for untargeted discovery of glycosylated compounds, might be of great use in this respect. Finally, further investigations on the xylosylation ability of GBA and the ability of GBA3 to degrade xylosylated compounds deserves attention.

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6

Summary

Within this thesis the central stage is taken by the discovery and investigation of transglycosylation of sterols. First, investigation focuses on the development of a method to accurately detect and quantify glucosylated metabolites in biological materials. Next, the studies concentrate on the formation and occurrence of specific glucosylated metabolites, in particular glucosyl-desmosterol (GlcDesm), glucosyl-7-dehydrocholesterol (Glc7DHC) and glucosylated vitamin D_3 ($GlcD_3$).

Chapter 1 introduces lysosomes and lysosomal diseases. Specific attention is paid to the retaining lysosomal β-glucosidase named glucocerebrosidase (GBA). Inherited deficiency of this enzyme causes Gaucher disease, a relatively common lysosomal storage disorder characterized by accumulation of glucosylceramide, the simplest glycosphingolipid. The features of GBA are described with emphasis to catalysis. Besides hydrolysis, the enzyme is able at special conditions to transglucosylate, i.e., transfer a glucose from a substrate to a sterol acceptor such as cholesterol. Glucosylated cholesterol, ubiquitous in tissues, is formed in this manner. Next, two other cellular β-glucosidases, the membrane-associated nonlysosomal glucosylceramidase (GBA2) and broad-specific cytosolic β-glucosidase (GBA3) are introduced, and their known role is glycolipid metabolism is discussed. The introductory chapter is concluded by discussing the physiological relevance of transglycosylation and the possible existence of additional acceptors in transglucosylation. Attention in this respect is focused to other sterols besides cholesterol.

Chapter 2 describes the development of a (sensitive) LC-MS/MS method to quantify glucosylated metabolites in natural materials like cell and tissue extracts and plasma. Attention was paid to optimization of the extraction procedure and the LC-MS/MS detection. Use was made of synthesized ^{13}C-encoded standards for accurate and sensitive quantification of glucosylated metabolites. Attention in this respect was focused to GlcChol, GlcDesm (glucosylated desmosterol) and Glc7DHC (glucosylated-7-dehydrocholesteol). The study led to a convenient and sensitive method for quantifying GlcChol, GlcDesm and Glc7DHC in biological materials.

The glucosylated lipids were extracted by two consecutive extraction methods, Bligh and Dyer followed by butanol/water extraction before analysis by LC-MS/MS. The chapter focusses on LC-MS/MS method development, commencing with discussing linearity of the calibration curve, together with the corresponding limit of detection (LOD), limit of quantification (LOQ) and signal-to-noise ratios (S/N). This was followed by mass spectrometric analysis, fragmentation and elution spectrum of the lipids of interest. Furthermore, intra/inter assay variation and carryover of the lipids was investigated. Next, storage of the glucosylated lipids within biological material (skin, plasma, breastmilk) was checked. Impurities in the lipid standards were investigated, as well. Last, detection of the glucosylated lipids within biological samples was shown.

Chapter 3 reports on the formation, degradation and occurrence of GlcDesm, a metabolite closely resembling GlcChol that indeed undergoes comparable modification with glucose. The study revealed the *in vitro* formation and degradation of GlcDesm by GBA and GBA2 from a β-glucoside donor and desmosterol. Furthermore, in human spleen the natural presence of GlcDesm was demonstrated. Gaucher disease (GD) patients deficient in GBA, show elevated levels of GlcDesm. Given the similarity of desmosterol and cholesterol, glucosylation of the former metabolite is not unexpected. It can be concluded from the findings that GBA plays largely a role in lysosomal degradation of GlcDesm and that its synthesis is mediated by GBA2.

Chapter 4 reports on the investigation of glucosylation of 7-dehydrocholesterol (7DHC) and its metabolite vitamin D_3. The formation, degradation and natural occurrence of the glucosylated metabolites Glc7DHC and $GlcD_3$ was studied. *In vitro* formation of Glc7DHC by GBA and GBA2 was observed. Degradation was only detectable for Glc7DHC by GBA. The natural occurrence of Glc7DHC in spleen and skin was observed. In the case of $GlcD_3$ *in vitro* formation by GBA and GBA2 was also demonstrable. In addition, conversion of Glc7DHC into $GlcD_3$ by UVB irradiation was observed.

Chapter 5 considers the ability of the broad-specific β-glucosidase (GBA3) to hydrolyze and transglucosylate. Studies on GBA3-mediated formation of GlcChol as well as degradation of GlcChol, Glc7DHC, $GlcD_3$ and GlcDesm were negative. A firm conclusion that GBA3 is not active towards the studied metabolites should however be further substantiated by experiments using a purified recombinant GBA3 in larger amounts.

Chapter 6 provides a general discussion of the work described in this thesis and an outlook. It discusses additional metabolites subject to transglycosylation to be explored, focusing on cholesterol derivatives, such as oxysterols, steroid hormones and bile acids. Next, the possible biological function and the pathophysiological relevance of glucosylated metabolites and acceptor abnormality is discussed. Proposed future research concerns untargeted discovery of glycosylated metabolites, by the use of a so-called 'transbody' (a modified glucose donor) and formation and occurrence of xylosylated metabolites. In conclusion, the work presented in this thesis gives several new opportunities for research.

Addendum I is a published paper on previous work on transglucosylation of GlcChol within mammals [1]. The paper reports on the formation and degradation of GlcChol by GBA and GBA2. The presented work of this publication initiated the research of this thesis.

Addendum II contains a published paper on the ability of GBA to transxylosylate, forming xylosyl-cholesterol [2]. This work prompts to investigate the formation and degradation of xylosylated metabolites, such as xylosyl-desmosterol, xylosyl-7-dehydrocholesterol or xylosyl-vitamin D_3.

References

1. Marques, A.R., Mirzaian, M., Akiyama, H., et al., *Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular beta-glucosidases.* J Lipid Res, 2016.
2. Boer, D.E., et al., *Human glucocerebrosidase mediates formation of xylosyl-cholesterol by β-xylosidase and transxylosidase reactions.* J Lipid Res, 2021. **62**: p. 100018.

Samenvatting

In deze thesis staat het ontdekken en onderzoeken van transglycosylering van sterolen centraal. Allereerst, ligt de onderzoeksfocus op de ontwikkeling van een methode waarmee accuraat glucosyleerde metaboliten gedetecteerd en gekwantificeerd kunnen worden, in biologisch materiaal. Vervolgens, wordt er geconcentreerd op het bestuderen van de formatie en aanwezigheid van specifieke geglucosyleerde metaboliten, in het speciaal glucosyl-desmosterol (GlcDesm), glucosyl-7-dehydrocholesterol (Glc7DHC) en geglucosyleerd vitamin D_3 ($GlcD_3$).

Hoofdstuk 1 introduceert lysosomen en lysosomale ziekten. Speciale aandacht wordt geschonken aan de lysosomale β-glucosidase genaamd glucocerebrosidase (GBA). Een overerfbare deficiëntie in dit enzym is oorzaak van de ziekte van Gaucher, een relatieve veel voorkomende lysosomale stapelingsziekte, gekarakteriseerd door de stapeling van glucosylceramide, de meest eenvoudige glycosphingolipide. De eigenschappen van GBA worden beschreven, met nadruk op de katalytische functie. Naast hydrolyse, is het enzym in staat om onder speciale omstandigheden te transglucosyleren, d.w.z., overdracht van glucose van een substraat naar een sterol acceptor zoals cholesterol. Glucosyleerd cholesterol, alom vertegenwoordigd in weefsels, wordt op deze manier gevormd. Vervolgens, worden twee andere cellulaire β-glucosidases, de membraan-geassocieerde nonlysosomale glucosylceramidase (GBA2) en de breed-specifice cytosolische β-glucosidase (GBA3) geïntroduceerd, en hun rol in glycolipide metabolisme wordt bediscussieerd. Het introducerende hoofdstuk sluit af met een discussie over de fysiologische relevantie van transglycosylatie en het mogelijke bestaan van additionele acceptoren voor transglucosylatie. Hierbij wordt aandacht geschonken aan andere sterolen naast cholesterol.

Hoofdstuk 2 beschrijft de ontwikkeling van een (sensitieve) LC-MS/MS methode voor het kwantificeren van geglucosyleerde metabolieten in natuurlijke materialen zoals cel- en weefsel extracten en plasma. Hierbij wordt aandacht geschonken aan de optimalisatie van de extractie procedure en de LC-MS/MS detectie. Voor de accurate en sensitieve kwantificatie van de geglucosyleerde metabolieten is gebruik gemaakt van gesynthetiseerde ^{13}C-gelabelde standaarden. De aandacht is hier gefocust op GlcChol, GlcDesm (geglucosyleerd desmosterol) en Glc7DHC (geglucosyleerd-7-dehydrocholesteol). Deze studie heeft geleidt tot een bruikbare en gevoelige methode voor het kwantificeren van GlcChol, GlcDesm en Glc7DHC in biologisch materiaal.

De geglucosyleerde lipiden werden geëxtraheerd met behulp van twee opeenvolgende extractie methodes, Bligh and Dyer gevolgd door een butanol/water extractie vooraf aan de analyse met LC-MS/MS. Dit hoofdstuk focust op LC-MS/MS methode ontwikkeling, beginnend met bediscussiëring van lineariteit van de calibratie curve, samen met de corresponderende limiet van detectie

(LVD), limiet van kwantificatie (LVK) en de signaal-ruis ratio (S/R). Gevolgd door massa spectrometrische analyse, fragmentatie en elutie spectrums van de lipiden van interesse. Tevens, intra/inter assay variatie en carryover van de lipiden is onderzocht. Vervolgens, is de opslag van geglucosyleerde lipiden in biologische material (huid, plasma, borstvoeding) gecontroleerd. Onzuiverheden in de lipidestandarden zijn ook onderzocht. Als laatst, wordt de detectie van de geglucosyleerde lipiden in biologische monsters getoond.

Hoofdstuk 3 rapporteert de vorming, degradatie en aanwezigheid van GlcDesm, een metaboliet sterk lijkend op GlcChol welke daadwerkelijk een vergelijkbare modificatie ondergaat met glucose. De studie onthult de *in vitro* vorming en degradatie van GlcDesm door GBA en GBA2 vanuit een β-glucoside donor en desmosterol. Bovendien, de natuurlijke aanwezigheid van GlcDesm in humane milt werd gedemonstreerd. Patiënten met de ziekte van Gaucher deficiënt in GBA, laten verhoogde waarden van GlcDesm. Gegeven de overeenkomst tussen desmosterol en cholesterol, is de transglucosylering van eerstgenoemde metaboliet niet onverwacht. Aan de hand van de bevindingen kan er geconcludeerd worden dat GBA een belangrijke rol speelt in lysosomale afbraak van GlcDesm en dat de synthese bemiddeld wordt door GBA2.

Hoofdstuk 4 rapporteert het onderzoek naar glucoylering van 7-dehydrocholesterol (7DHC) en zijn metaboliet vitamine D_3. De vorming, degradatie en natuurlijke aanwezigheid van de geglucosyleerde metabolieten Glc7DHC en $GlcD_3$ werd bestudeerd. *In vitro* vorming van Glc7DHC door GBA en GBA2 werd geobserveerd. Degradatie werd alleen gedetecteerd voor Glc7DHC door GBA. De natuurlijke aanwezigheid van Glc7DHC in milt en huid werd waargenomen. In het geval van $GlcD_3$ werd vorming door GBA en GBA2 ook gedemonstreerd. Bijkomend werd de omzetting van Glc7DHC in $GlcD_3$ onder invloed van UVB straling waargenomen.

Hoofdstuk 5 beschouwd het vermogen van de breed-specifieke β-glucosidase (GBA3) om te hydrolyseren en transglucosyleren. Onderzoeken naar GBA3-gemedieerde vorming van GlcChol evenals degradatie van GlcChol, Glc7DHC, $GlcD_3$ en GlcDesm waren negatief. Een krachtige conclusie dat GBA3 niet actief is jegens de bestudeerde metabolieten moet met verdere experimenten met zuivere recombinante GBA3 in hogere hoeveelheden waargemaakt worden.

Hoofdstuk 6 voorziet in een algemene discussie over het werk in deze thesis en een toekomst visie. Het bediscussieerd de verkenning naar aanvullende metabolieten die onderworpen kunnen worden aan transglycosylatie, met de focus op afgeleiden van cholesterol, zoals oxysterolen, steroid hormonen en galzuren. Vervolgens, een mogelijke biologische functie en pathofysiologische relevantie van de geglucosyleerde metabolieten en afwijkingen in acceptoren wordt bediscussieerd. Toekomstig onderzoek betreft ongerichte ontdekking van

geglycosyleerde metabolieten, door gebruik te maken van een zogenoemde 'transbody' (een gemodificeerde glucose donor) en de vorming en aanwezigheid van gexylosyleerde metabolieten. Concluderend, het werk gepresenteert in deze thesis beschrijft verscheidene nieuw richtingen voor potentieel onderzoek.

Addendum I is een gepubliceerd artikel over voormalig werk over transglucosylering van GlcChol in zoogdieren [1]. Het artikel rapporteert over de vorming en afbraak van GlcChol door GBA en GBA2. Het gepresenteerde werk in deze publicatie initieert het onderzoek in deze thesis.

Addendum II bevat een gepubliceerd artikel over de capaciteit van GBA om te transxylosyleren, vorming xylosyl-cholsterol [2]. Dit werk spoort aan tot het onderzoeken van de vorming en afbraak van gexylosyleerde metabolieten, zoals xylosyl-desmosterol, xylosyl-7-dehydrocholesterol of xylosyl-vitamin D_3.

Bronnen

1. Marques, A.R., Mirzaian, M., Akiyama, H., et al., *Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular beta-glucosidases.* J Lipid Res, 2016.
2. Boer, D.E., et al., *Human glucocerebrosidase mediates formation of xylosyl-cholesterol by β-xylosidase and transxylosidase reactions*. J Lipid Res, 2021. **62**: p. 100018.

About the author – Curriculum Vitae

I was born in Goes, the Netherlands on May 2nd 1990. My secondary education was at Het Goese Lyceum, including subjects such as biology, chemistry, physics, mathematics and the international Baccalaureate English, IB certificate English. In 2009, I started the bachelor Life Science and Technology at Leiden University and Delft University of Technology. During my bachelor thesis I had the opportunity to work at the Leiden University Medical Centre, at the department of diagnostics. Here I worked on my first, disease and patient related project. During the internship I developed an analysis pipeline for Whole Exome Sequencing (WES) data and a Single Nucleotide Polymorphism (SNP) validation test for Next Generation Sequencing (NGS) sample identification. The developed validation allows detection of sample switches. The used analysis method was a Melting Curve Analysis to determine the genotype of the samples. After this internship I realized that I wanted to do more research, so I decided to start the Research and Development master of Life Science and Technology at University of Leiden in 2013.

During my master thesis, I did my internship at the Leiden University Medical Centre, department of Human Genetics – Polycystic Kidney Disease. The research group focuses on Autosomal Dominant Polycystic Kidney Disease. The disease is characterized by enlargement of the kidneys and occurrence of large fluid filled cysts, which results in progressive deterioration of kidney function. The disease is caused by mutations in the PKD1 or PKD2 genes, encoding polycystin-1 (PC1) and polycystin-2 (PC2). When either one of these proteins is lost a complex network of signaling pathways is disrupted. The PKD research group researches the signaling pathways involved in PKD, with the aim to develop treatment. During this research project I worked on signaling pathways in Polycystic Kidney Disease (a kidney disease where many fluid filled cysts destroy renal function). The focus of my project was on two important transcriptional coactivators, YAP and TAZ. These proteins are potential modulators in Polycystic Kidney Disease. During the project I studied the expression levels of YAP and TAZ on RNA and protein level during disease progression and worked with antisense oligonucleotide (ASOs) technology, in order to down-regulate YAP and TAZ expression. During the project I practiced my skills in culturing and transfecting cells, qPCR and Western blot analysis. I also developed skills in immunofluorescence techniques, when I studied the localisation of the proteins within cells.

While doing my master, I worked at 'Reizend DNAlab', an organization that organizes specialized practicals for high school students. The main topic of these practicals was protein folding and disease. During these practicals the students perform immunofluorescence experiments, and depending on the educational level, study protein function or create protein crystals. This job showed me that I have, besides research, a passion for education. Therefore, I applied in 2015 for this special PhD program, where I could combine both research and teaching. During this PhD program I did my research and work of this thesis at the Leiden Institute of Chemistry, in the Medical Biochemistry department under the supervision of Prof. dr. J.M.F.G. Aerts. Meanwhile I obtained my Master of Education - first degree chemistry teacher at Interfacultair Centrum voor Lerarenopleiding (ICLON) – Leiden University. My thesis for my master of Education covered research on cooperative learning for high school chemistry students. The research was nominated for the ROS Rijnland research price, and received the second price.

List of publications

1. Marques AR, Mirzaian M, Akiyama H, Wisse P, Ferraz MJ, Gaspar P, Ghauharali-van der Vlugt K, **Meijer R**, Giraldo P, Alfonso P, Irún P, Dahl M, Karlsson S, Pavlova EV, Cox TM, Scheij S, Verhoek M, Ottenhoff R, van Roomen CP, Pannu NS, van Eijk M, Dekker N, Boot RG, Overkleeft HS, Blommaart E, Hirabayashi Y, Aerts JM. **Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular β-glucosidases.** J Lipid Res. 2016 Mar;57(3):451-63. Doi: 10.1194/jlr.M064923. Epub 2016 Jan 2. PMID: 26724485; PMCID: PMC4766994.

2. Boer DEC, Mirzaian M, Ferraz MJ, Zwiers KC, Baks MV, Hazeu MD, Ottenhoff R, Marques ARA, **Meijer R**, Roos JCP, Cox TM, Boot RG, Pannu N, Overkleeft HS, Artola M, Aerts JM. **Human glucocerebrosidase mediates formation of xylosyl-cholesterol by β-xylosidase and transxylosidase reactions.** J Lipid Res. 2021;62:100018. Doi: 10.1194/jlr.RA120001043. Epub 2021 Jan 6. PMID: 33361282; PMCID: PMC7903134.

3. Aerts JM, Ferraz MJ, Mirzaian M, Gaspar P, van Oussoren S, Wisse P, Kuo CL, Lelieveld LT, Kytidou K, Hazeu MD, Boer DEC, **Meijer R**, van der Lienden MJC Lienden, Chao DHM, Gabriel TL, Aten J, Overkleeft HS, van Eijk M, Boot RG, Marques ARA.**Lysosomal Storage Diseases. For Better or Worse: Adapting to Defective Lysosomal Glycosphingolipid Breakdown.** eLs John Wiley & Sons, Ltd., Chichester, UK, 2017, pp. 1-13.

Addendum I

Marques AR, Mirzaian M, Akiyama H, Wisse P, Ferraz MJ, Gaspar P, Ghauharali-van der Vlugt K, **Meijer R**, Giraldo P, Alfonso P, Irún P, Dahl M, Karlsson S, Pavlova EV, Cox TM, Scheij S, Verhoek M, Ottenhoff R, van Roomen CP, Pannu NS, van Eijk M, Dekker N, Boot RG, Overkleeft HS, Blommaart E, Hirabayashi Y, Aerts JM. **Glucosylated cholesterol in mammalian cells and tissues: formation and degradation by multiple cellular β-glucosidases.** J Lipid Res. 2016 Mar;57(3):451-63. Doi: 10.1194/jlr.M064923. Epub 2016 Jan 2. PMID: 26724485; PMCID: PMC4766994.

The membrane lipid glucosylceramide (GlcCer) is continuously formed and degraded. Cells express two GlcCer-degrading β-glucosidases, GBA and GBA2, located in and outside the lysosome, respectively. Here we demonstrate that through transglucosylation both GBA and GBA2 are able to catalyze *in vitro* the transfer of glucosyl-moieties from GlcCer to cholesterol, and vice versa. Furthermore, the natural occurrence of 1-O-cholesteryl-β-D-glucopyranoside (GlcChol) in mouse tissues and human plasma is demonstrated using LC-MS/MS and $^{13}C_6$-labelled GlcChol as internal standard. In cells the inhibition of GBA increases GlcChol, whereas inhibition of GBA2 decreases glucosylated sterol. Similarly, in GBA2-deficient mice GlcChol is reduced. Depletion of GlcCer by inhibition of GlcCer synthase decreases GlcChol in cells and likewise in plasma of inhibitor-treated Gaucher disease patients. In tissues of mice with Niemann-Pick type C, a condition characterized by intralysosomal accumulation of cholesterol, marked elevations in GlcChol occur as well. When lysosomal accumulation of cholesterol is induced in cultured cells, GlcChol is formed via lysosomal GBA. This illustrates that reversible transglucosylation reactions are highly dependent on local availability of suitable acceptors. In conclusion, mammalian tissues contain GlcChol formed by transglucosylation through β-glucosidases using GlcCer as donor. Our findings reveal a novel metabolic function for GlcCer.

Addendum II

Boer DE, Mirzaian M, Ferraz MJ, Zwiers KC, Baks MV, Hazeu MD, Ottenhoff R, Marques ARA, **Meijer R**, Roos JCP, Cox TM, Boot RG, Pannu N, Overkleeft HS, Artola M, Aerts JM. **Human glucocerebrosidase mediates formation of xylosyl-cholesterol by β-xylosidase and transxylosidase reactions.** J Lipid Res. 2021;62:100018. Doi: 10.1194/jlr.RA120001043. Epub 2021 Jan 6. PMID: 33361282; PMCID: PMC7903134.

Deficiency of glucocerebrosidase (GBA), a lysosomal β-glucosidase, causes Gaucher disease. The enzyme hydrolyzes β-glucosidic substrates and transglucosylates cholesterol to cholesterol-β-glucoside. Here we show that recombinant human GBA also cleaves β-xylosides and transxylosylates cholesterol. The xylosyl-cholesterol formed acts as acceptor for subsequent formation of di-xylosylcholesterol. Common mutant forms of GBA from patients with Gaucher disease with reduced β-glucosidase activity were similarly impaired in β-xylosidase, transglucosidase and transxylosidase activities, except for a slightly reduced xylosidase/glucosidase activity ratio of N370S GBA and a slightly reduced transglucosylation/glucosidase activity ratio of D409H GBA. XylChol was found to be reduced in spleen from Gaucher disease patients. The origin of newly identified XylChol in mouse and human tissues was investigated. Cultured human cells exposed to exogenous β-xylosides generated XylChol in a manner dependent on active lysosomal GBA but not the cytosol-facing β-glucosidase GBA2. We later sought an endogenous β-xyloside acting as donor in transxylosylation reactions, identifying xylosylated ceramide (XylCer) in cells and tissues that serve as donor in the formation of XylChol. UDPglucosylceramide synthase (GCS) was unable to synthesize XylChol but could catalyse formation of XylCer. Thus, food-derived β-D-xyloside and XylCer are potential donors for the GBA-mediated formation of XylChol in cells. The enzyme GCS produces XylCer at a low rate. Our findings point to further catalytic versatility of GBA and prompt a systematic exploration of the distribution and role of xylosylated lipids.

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Next I would like to thank my supervisor and promotor, Prof. dr. Hans Aerts. As he gave me the opportunity to work in his research group and he made me a better researcher by supervising and discussing my research. Also would like to thank my co-promotor dr. Rolf Boot, for the good talks at the end of my PhD. Also many thanks to dr. Marta Artola, for all the support in reading my work and correcting my chemical structures.

I believe that research takes teamwork to become good research. Therefore I would like to thank the people that helped making my research better.

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