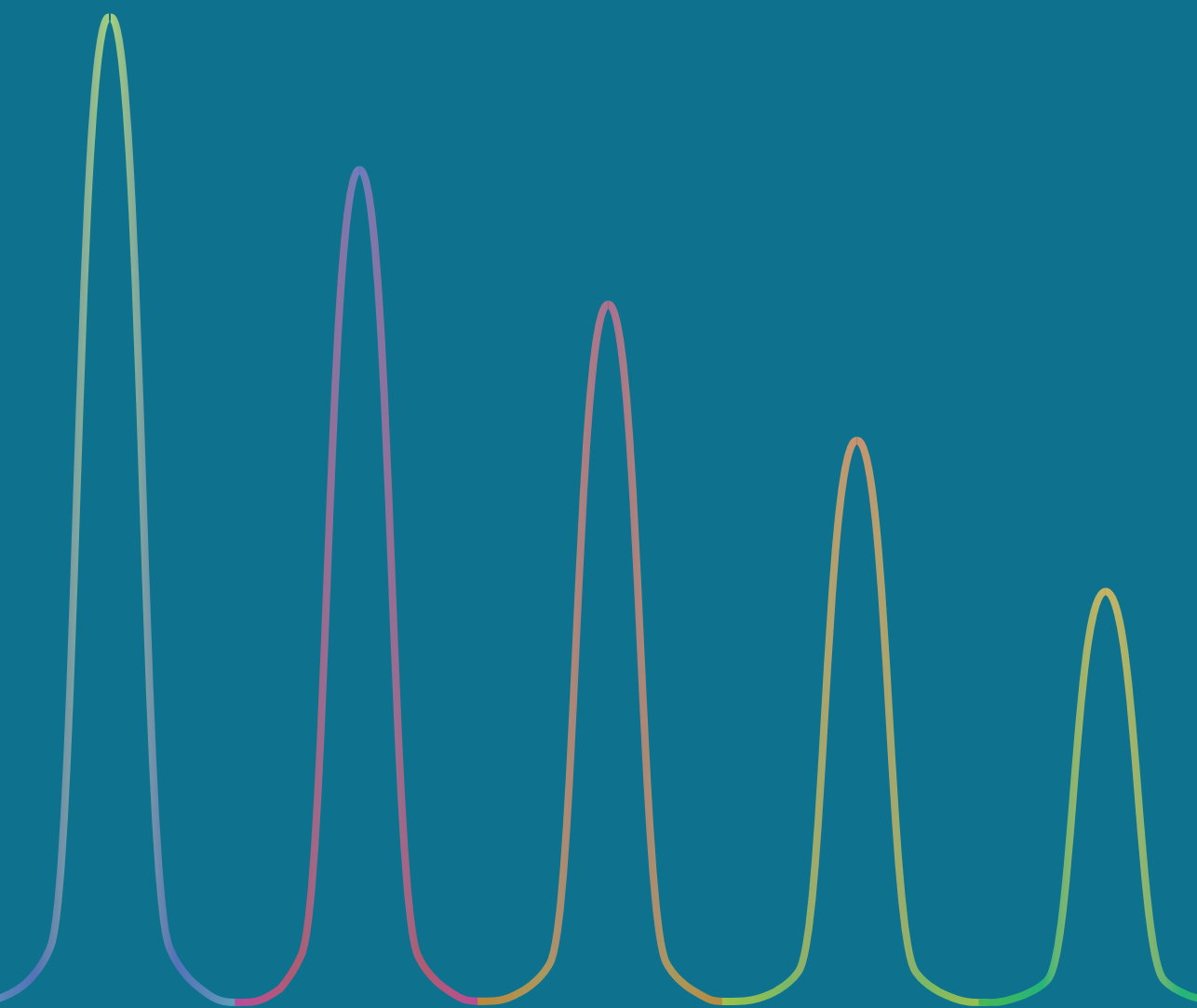
Discovery and characterization of new glucosylated metabolites: pathophysiological consequences

Meijer, H.N.J.



Chapter 1

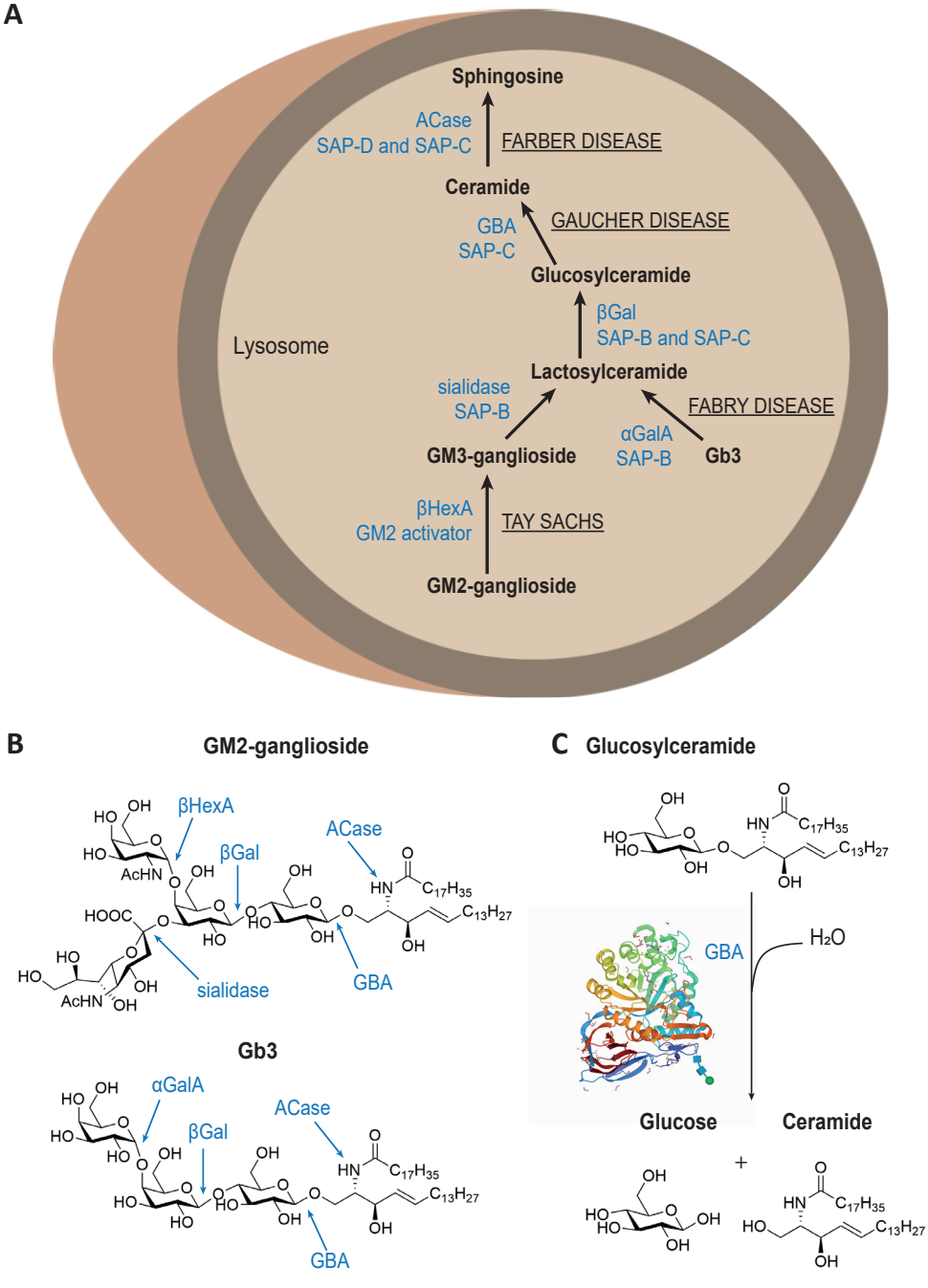
Introduction and outline

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].

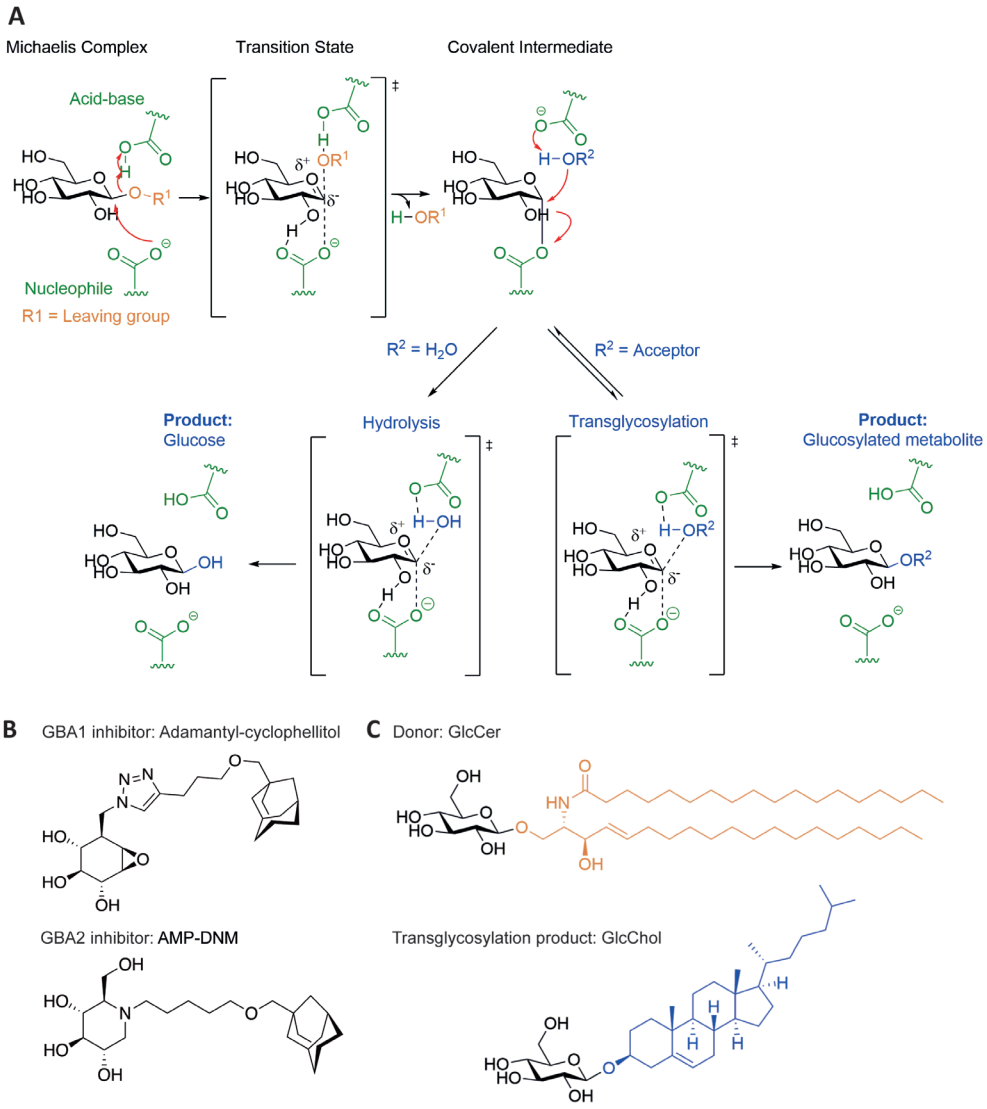


Figure 2. Reaction mechanism, inhibitors and transglycosylation.

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].

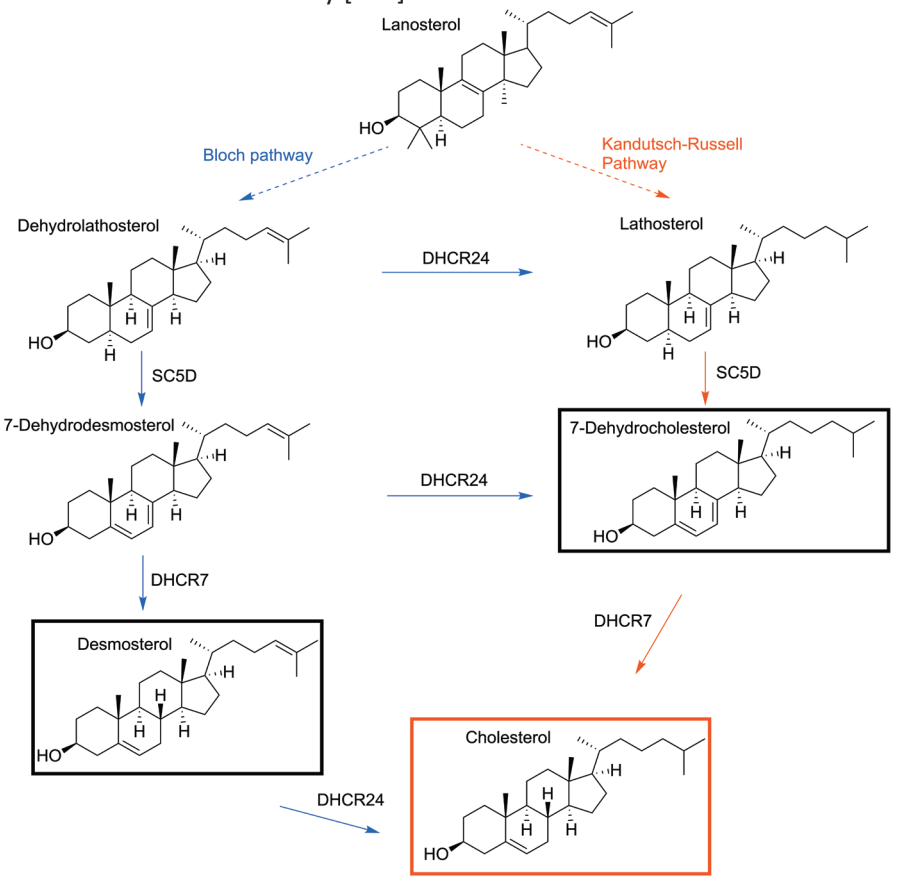


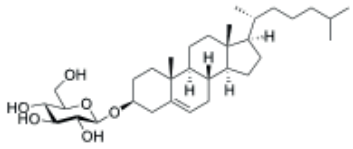
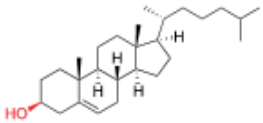
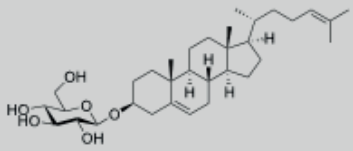
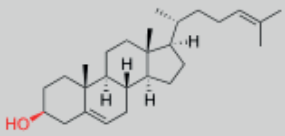
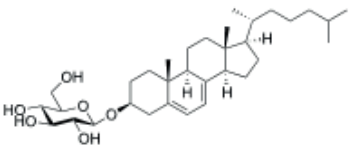
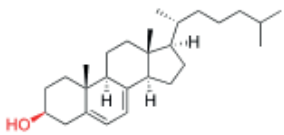
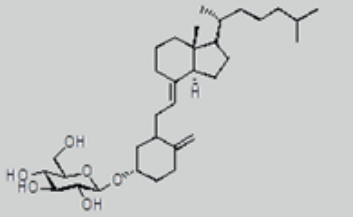
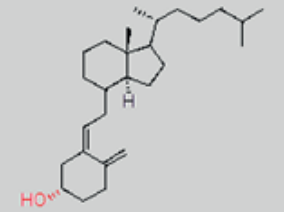
Figure 3. Cholesterol pathways.

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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