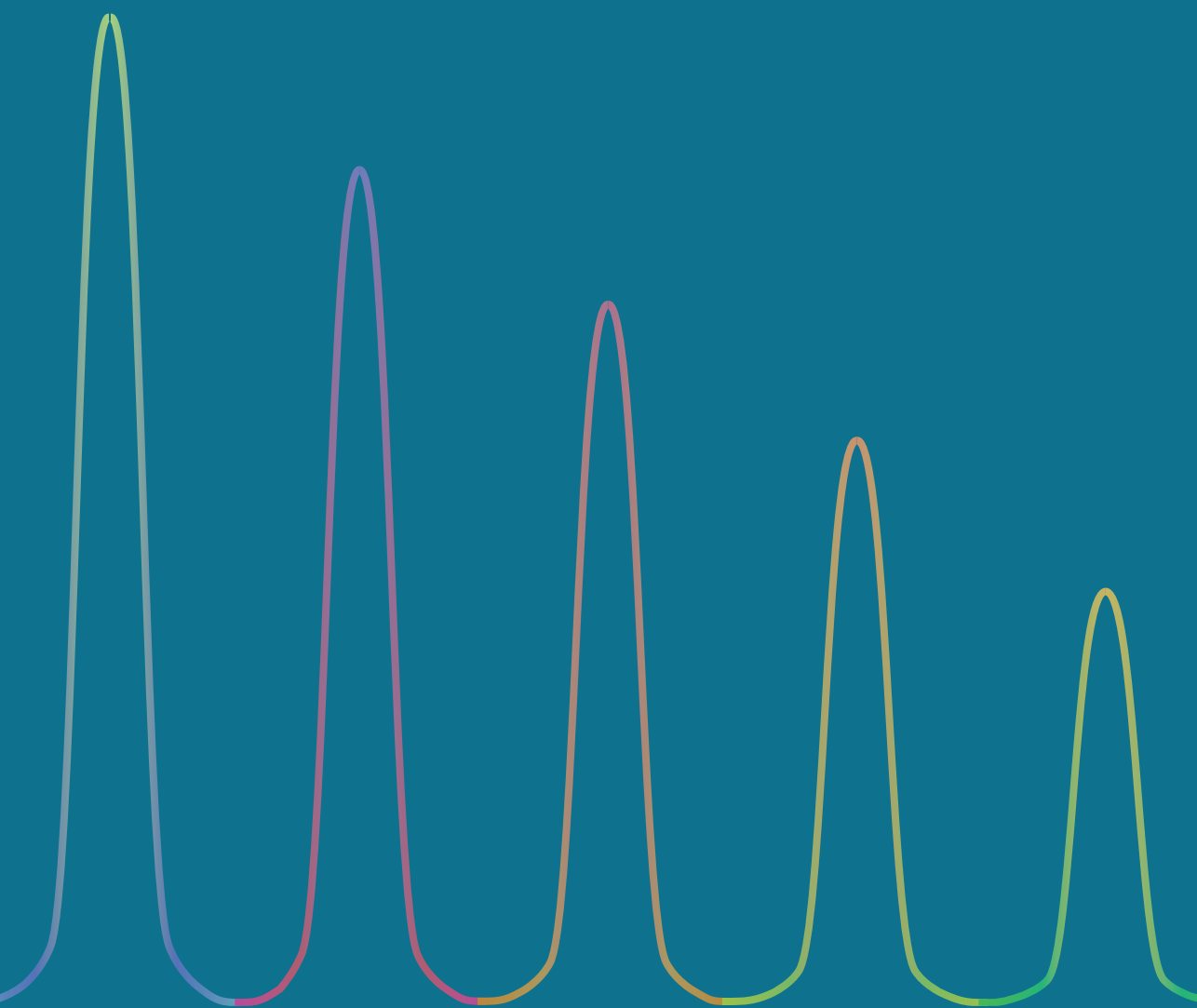
Discovery and characterization of new glucosylated metabolites: pathophysiological consequences

Meijer, H.N.J.



Chapter 3

Formation and degradation of glucosyl-desmosterol

To be submitted in revised form

Chapter 3 - Formation and degradation of glucosyl-desmosterol

Rianne Meijer, Ken Kok, Patrick Wisse, Mina Mirzaian, Marta Artola, Johannes M. Aerts.

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.

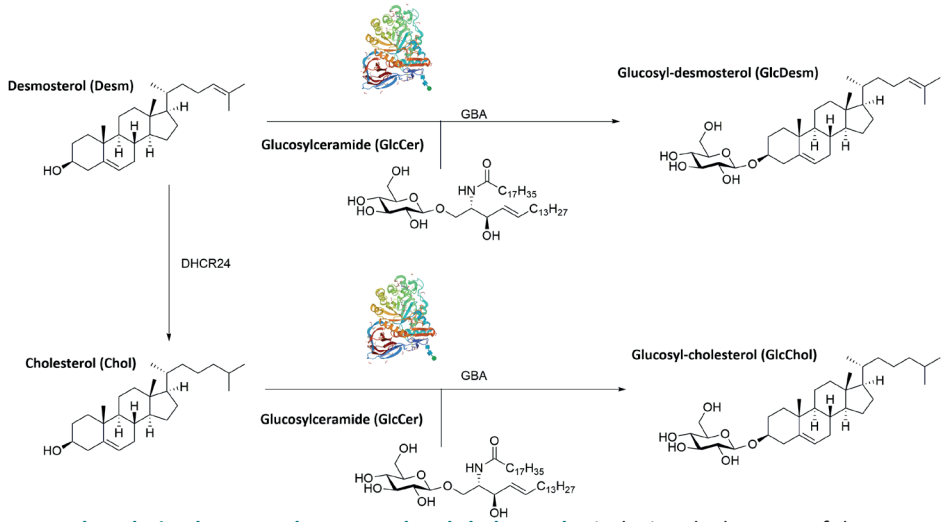


Figure 1 The relation between desmosterol and cholesterol. *Displaying the last step of the Bloch pathway in which desmosterol (Desm) is converted into cholesterol (Chol) by the enzyme 24-Dehydrocholesterol Reductase (DHCR24). Also includes the transglucosylation reaction by the enzyme Glucocerebrosidase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

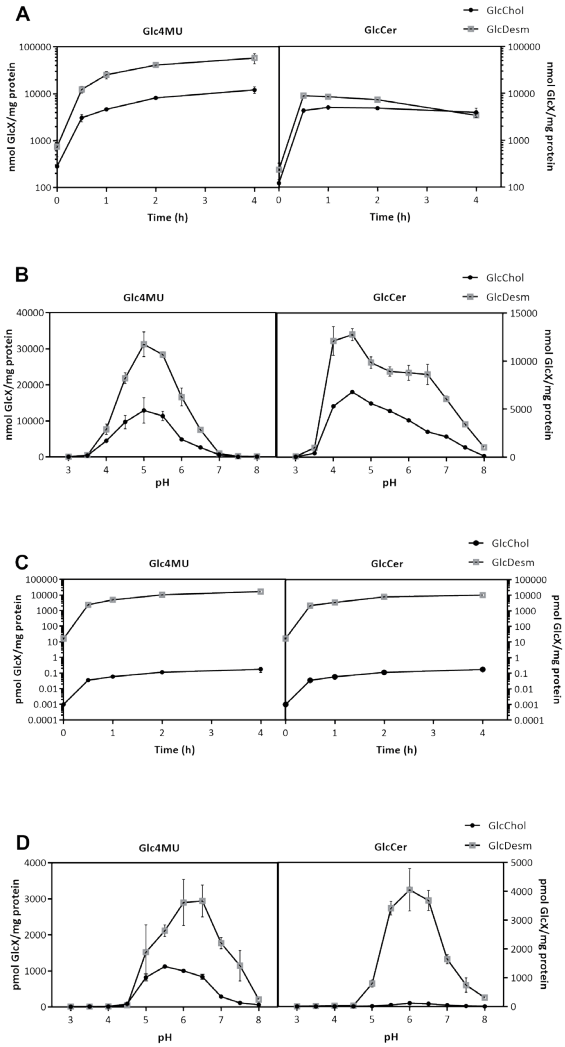


Figure 2. In vitro formation of GlcDesm. *A) rGBA was incubated for varied times with cholesterol (Chol) or desmosterol (Desm) in the presence of 4-methylumbelliferyl ß-D-glucopyranoside (4MUGlc) or C18:1-GlcCer (GlcCer) donor. Formation of GlcChol and GlcDesm was measured by LC-MS/MS (nmol/mg protein).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

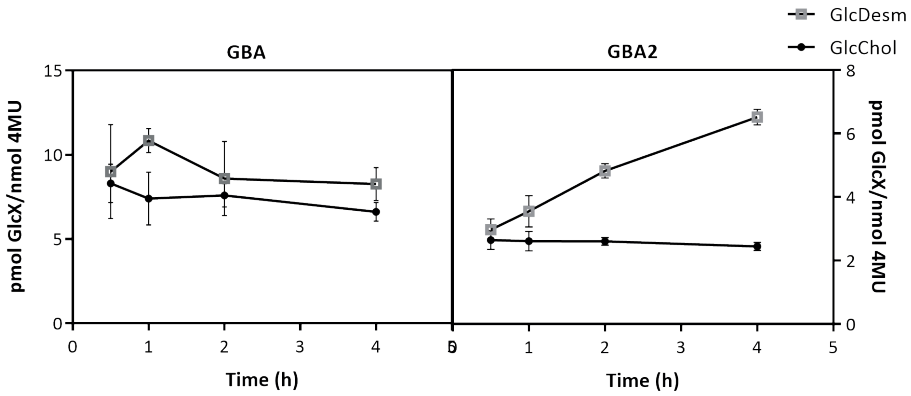


Figure 3. Ratio Transglucosylation and hydrolase activities. *rGBA was incubated for varied times with cholesterol (Chol) or Desmosterol (Desm) in the presence of 4-methylumbelliferyl β-D-glucopyranoside (4MUGlc). 4MU-emitted fluorescence (nmol 4MU/mg protein) was measured with a LS-55 Fluorescence spectrometer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

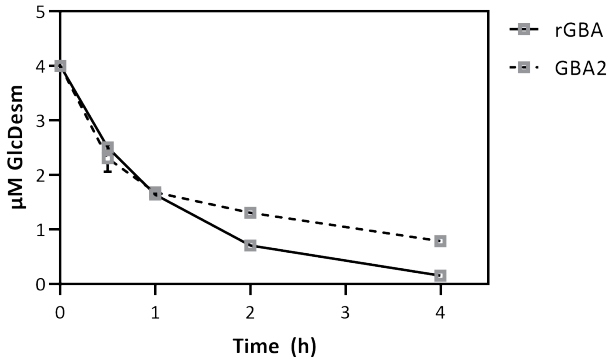


Figure 4. Degradation of GlcDesm. *A) Either rGBA or GBA2 was incubated for varied times with 4 µM GlcDesm. Degradation of GlcDesm was measured by LC-MS/MS (µM) and corrected for amount of protein.*

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.

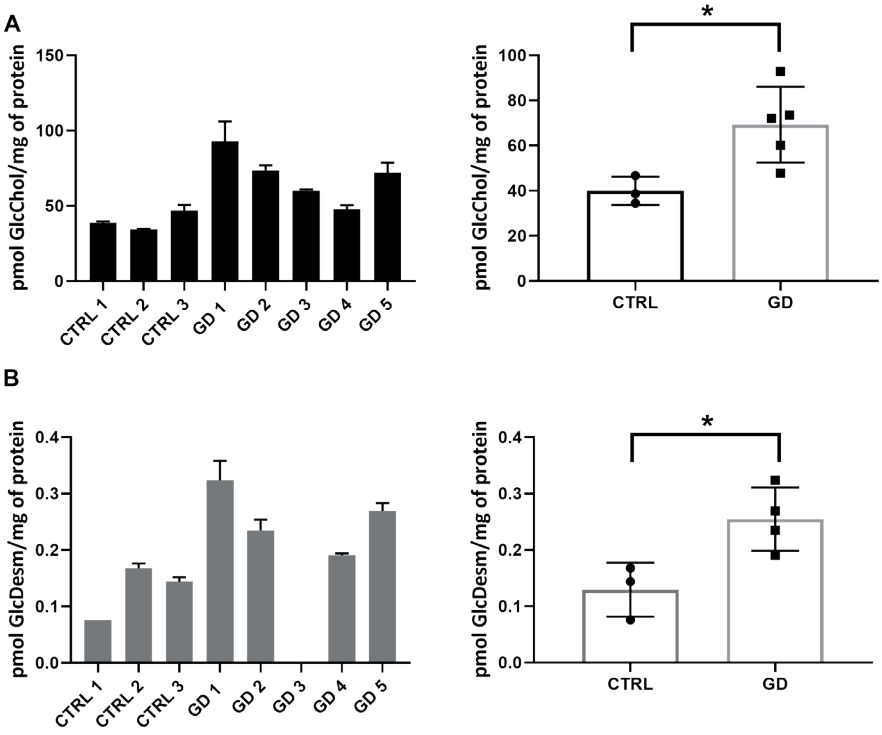


Figure 5. Levels of GlcChol and GlcDesm in GD spleen. *LC-MS/MS analysis of GlcChol and GlcDesm occurrence in human control spleens and human GD spleens. Errors bars are standard deviation of technical duplicates. Data were analysed by unpaired Student's t-test. P values <0.05 were considered significant (*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.

Acknowledgements
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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).

References

1. Stokes, W.M., W.A. Fish, and F.C. Hickey, *Metabolism of cholesterol in the chick embryo.* I. The synthesis of cholesterol. J Biol Chem, 1953. **200**(2): p. 683-9.
2. Stokes, W.M., W.A. Fish, and F.C. Hickey, *Metabolism of cholesterol in the chick embryo. II. Isolation and chemical nature of two companion sterols.* J Biol Chem, 1956. **220**(1): p. 415-30.
3. Bloch, K., R.B. Clayton, and P.B. Schneider, *Synthesis of lanosterol in vivo.* J Biol Chem, 1957. **224**(1): p. 175-83.
4. Johnston, J.D. and K. Bloch, *In Vitro Conversion of Zymosterol and Dihydrozymosterol to Cholesterol.* Journal of the American Chemical Society, 1957. **79**(5): p. 1145-1149.
5. Stokes, W.M., F.C. Hickey, and W.A. Fish, *Sterol metabolism. I. The occurrence of desmosterol (24-dehydrocholesterol) in rat skin and its conversion in vivo to cholesterol.* J Biol Chem, 1958. **232**(1): p. 347-59.
6. Roux, C., *Teratogenic action of Triparanol in animals.* Arch Fr Pediatr, 1964. **21**: p. 451-64.
7. FitzPatrick, D.R., et al., *Clinical phenotype of desmosterolosis.* Am J Med Genet, 1998. **75**(2): p. 145-52.
8. Honda, A., et al., *Defective conversion of 7-dehydrocholesterol to cholesterol in cultured skin fibroblasts from Smith-Lemli-Opitz syndrome homozygotes.* J Lipid Res, 1995. **36**(7): p. 1595-601.
9. Tint, G.S., et al., *Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome.* N Engl J Med, 1994. **330**(2): p. 107-13.
10. Croce, C.M., et al., *Restoration of the conversion of desmosterol to cholesterol in L-cells after hybridization with human fibroblasts.* Proc Natl Acad Sci U S A, 1974. **71**(1): p. 110-3.
11. Waterham, H.R., et al., *Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis.* Am J Hum Genet, 2001. **69**(4): p. 685-94.
12. Andersson, H.C., L. Kratz, and R. Kelley, *Desmosterolosis presenting with multiple congenital anomalies and profound developmental delay.* Am J Med Genet, 2002. **113**(4): p. 315-9.
13. Zolotushko, J., et al., T*he desmosterolosis phenotype: spasticity, microcephaly and micrognathia with agenesis of corpus callosum and loss of white matter.* Eur J Hum Genet, 2011. **19(**9): p. 942-6.
14. Schaaf, C.P., et al., *Desmosterolosis-phenotypic and molecular characterization of a third case and review of the literature.* American journal of medical genetics. Part A, 2011. **155A(**7): p. 1597-1604.
15. Lin, D.S., et al., *Unique lipids of primate spermatozoa: desmosterol and docosahexaenoic acid.* J Lipid Res, 1993. **34**(3): p. 491-9.
16. Mutka, A.L., et al., *Secretion of sterols and the NPC2 protein from primary astrocytes.* J Biol Chem, 2004. **279**(47): p. 48654-62.

17. Rothblat, G.H., et al., *Desmosterol as the major sterol in L-cell mouse fibroblasts grown in sterol-free culture medium.* Science, 1970. **169**(3948): p. 880-2.
18. Huster, D., et al., D*esmosterol may replace cholesterol in lipid membranes.* Biophys J, 2005. **88**(3): p. 1838-44.
19. Samuli Ollila, O.H., et al., *Role of sterol type on lateral pressure profiles of lipid membranes affecting membrane protein functionality: Comparison between cholesterol, desmosterol, 7-dehydrocholesterol and ketosterol.* J Struct Biol, 2007. **159**(2): p. 311-23.
20. Mirza, R., et al., *DHCR24 gene knockout mice demonstrate lethal dermopathy with differentiation and maturation defects in the epidermis.* J Invest Dermatol, 2006. **126**(3): p. 638-47.
21. Mirza, R., et al., *Increased expression of aquaporin-3 in the epidermis of DHCR24 knockout mice.* Br J Dermatol, 2008. **158**(4): p. 679-84.
22. Smigiel, R., et al., *Novel frameshifting mutations of the ZMPSTE24 gene in two siblings affected with restrictive dermopathy and review of the mutations described in the literature.* Am J Med Genet A, 2010. **152a**(2): p. 447-52.
23. Khanna, P., J.M. Opitz, and E. Gilbert-Barness, *Restrictive dermopathy: report and review.* Fetal Pediatr Pathol, 2008. **27**(2): p. 105-18.
24. Rodriguez-Acebes, S., et al., *Desmosterol can replace cholesterol in sustaining cell proliferation and regulating the SREBP pathway in a sterol-Delta24-reductase-deficient cell line.* Biochem J, 2009. **420**(2): p. 305-15.
25. Spann, N.J., et al., *Regulated accumulation of desmosterol integrates macrophage lipid metabolism and inflammatory responses.* Cell, 2012. **151**(1): p. 138-52.
26. Glass, C.K. and S. Ogawa, *Combinatorial roles of nuclear receptors in inflammation and immunity.* Nat Rev Immunol, 2006. **6**(1): p. 44-55.
27. Horton, J.D., J.L. Goldstein, and M.S. Brown, *SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver.* J Clin Invest, 2002. **109**(9): p. 1125-31.
28. Daimiel, L.A., et al., *Promoter analysis of the DHCR24 (3beta-hydroxysterol Delta(24)-reductase) gene: characterization of SREBP (sterol-regulatory-element-binding protein)-mediated activation.* Biosci Rep, 2012. **33**(1): p. 57-69.
29. Lehmann, J.M., et al., *Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway.* J Biol Chem, 1997. **272**(6): p. 3137-40.
30. Muse, E.D., et al., *Cell-specific discrimination of desmosterol and desmosterol mimetics confers selective regulation of LXR and SREBP in macrophages.* Proc Natl Acad Sci U S A, 2018. **115**(20): p. E4680-e4689.

31. Heverin, M., et al., *Studies on the cholesterol-free mouse: strong activation of LXR-regulated hepatic genes when replacing cholesterol with desmosterol.* Arterioscler Thromb Vasc Biol, 2007. **27**(10): p. 2191-7.
32. Peet, D.J., et al., *Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha.* Cell, 1998. **93**(5): p. 693-704.
33. Joseph, S.B., et al., *Synthetic LXR ligand inhibits the development of atherosclerosis in mice.* Proc Natl Acad Sci U S A, 2002. **99**(11): p. 7604-9.
34. Andersson, S., et al., *Inactivation of liver X receptor beta leads to adult-onset motor neuron degeneration in male mice.* Proc Natl Acad Sci U S A, 2005. **102**(10): p. 3857-62.
35. Fumagalli, R. and R. Paoletti, *The identification and significance of desmosterol in the developing human and animal brain.* Life Sci (1962), 1963. **5**: p. 291-5.
36. Dennick, R.G., P.D. Dean, and D.A. Abramovich, *Desmosterol levels in human foetal brain--a reassessment.* J Neurochem, 1973. **20**(4): p. 1293-4.
37. Tint, G.S., et al., *Desmosterol in brain is elevated because DHCR24 needs REST for Robust Expression but REST is poorly expressed.* Dev Neurosci, 2014. **36**(2): p. 132-42.
38. Jensen, R.G., *The lipids in human milk.* Prog Lipid Res, 1996. **35**(1): p. 53-92.
39. Beggio, M., et al., *Quantification of total cholesterol in human milk by gas chromatography.* J Sep Sci, 2018. **41**(8): p. 1805-1811.
40. Clark, R.M., et al., *Desmosterol in human milk.* Lipids, 1983. **18**(3): p. 264-6.
41. Galli, G., E.G. Paoletti, and J.F. Weiss, Sterol precursors of cholesterol in adult human brain. Science, 1968. 162(3861): p. 1495-6.
42. Sato, Y., et al., *Reduced plasma desmosterol-to-cholesterol ratio and longitudinal cognitive decline in Alzheimer's disease.* Alzheimers Dement (Amst), 2015. **1**(1): p. 67-74.
43. Wisniewski, T., K. Newman, and N.B. Javitt, *Alzheimer's disease: brain desmosterol levels.* J Alzheimers Dis, 2013. **33**(3): p. 881-8.
44. Lathe, R., A. Sapronova, and Y. Kotelevtsev, *Atherosclerosis and Alzheimer -diseases with a common cause? Inflammation, oxysterols, vasculature.* BMC geriatrics, 2014. **14**: p. 36-36.
45. Cermakova, P., et al., *Heart failure and Alzheimer's disease.* Journal of internal medicine, 2015. **277**(4): p. 406-425.
46. Avigan, J. and D. Steinberg, *Deposition of desmosterol in the lesions of experimental atherosclerosis.* Lancet, 1962. **1**(7229): p. 572.
47. Herndon, J.H., Jr. and M.D. Siperstein, *Desmosterol deposition in human and experimental atherosclerosis.* Circ Res, 1963. **12**: p. 228-34.
48. Hollander, W., A.V. Chobanian, and R.W. Wilkins, *The effects of triparanol (MER-29) in subjects with and without coronary artery disease.* Jama, 1960. **174**: p. 5-12.

49. 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.
50. Goldblatt, J., *Type I Gaucher disease.* J Med Genet, 1988. **25**(6): p. 415-8.
51. Sidransky, E., *New perspectives in type 2 Gaucher disease.* Adv Pediatr, 1997. **44**: p. 73-107.
52. Luk, E. and A.I. Gotlieb, *Atherosclerosis, in Pathobiology of Human Disease,* L.M. McManus and R.N. Mitchell, Editors. 2014, Academic Press: San Diego. p. 2970-2985.
53. de Fost, M., et al., *Low HDL cholesterol levels in type I Gaucher disease do not lead to an increased risk of cardiovascular disease.* Atherosclerosis, 2009. **204**(1): p. 267-72.
54. Lloyd-Evans, E., et al., *Inhibition of NPC1 function by cholesterol precursors in Smith–Lemli–Opitz syndrome induces an intracellular phenotype identical to Niemann–Pick C disease.* Chemistry and Physics of Lipids, 2009. **160**: p. S4.
55. Platt, F.M., et al., *Disorders of cholesterol metabolism and their unanticipated convergent mechanisms of disease.* Annu Rev Genomics Hum Genet, 2014. **15**: p. 173-94.
56. Holleran, W.M., et al., *Consequences of beta-glucocerebrosidase deficiency in epidermis. Ultrastructure and permeability barrier alterations in Gaucher disease.* J Clin Invest, 1994. **93**(4): p. 1756-64.
57. Sidransky, E., et al., *Epidermal abnormalities may distinguish type 2 from type 1 and type 3 of Gaucher disease.* Pediatr Res, 1996. **39**(1): p. 134-41.
58. Holleran, W.M., et al., P*rocessing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function.* J Clin Invest, 1993. **91**(4): p. 1656-64.
59. Chan, A., et al., *Skin ultrastructural findings in type 2 Gaucher disease: diagnostic implications.* Mol Genet Metab, 2011. **104**(4): p. 631-6.
60. Boer, D.E.C., et al., *Glucocerebrosidase: Functions in and Beyond the Lysosome.* J Clin Med, 2020. **9**(3).
61. Boer, D.E.C., et al., *Glucosylated cholesterol in skin: synthetic role of extracellular glucocerebrosidase.* Clin Chim Acta, 2020.
62. Artola, M., et al., *Functionalized Cyclophellitols Are Selective Glucocerebrosidase Inhibitors and Induce a Bona Fide Neuropathic Gaucher Model in Zebrafish.* J Am Chem Soc, 2019. **141**(10): p. 4214-4218.
63. Aerts, J.M., et al., *The occurrence of two immunologically distinguishable beta-glucocerebrosidases in human spleen.* Eur J Biochem, 1985. **150**(3): p. 565-74.
64. Boot, R.G., et al., *Identification of the non-lysosomal glucosylceramidase as beta-glucosidase 2.* J Biol Chem, 2007. **282**(2): p. 1305-12.
65. Bligh, E.G. and W.J. Dyer, *A rapid method of total lipid extraction and purification.* Can J Biochem Physiol, 1959. **37**(8): p. 911-7.