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Discovery and characterization of new glucosylated metabolites: pathophysiological consequences

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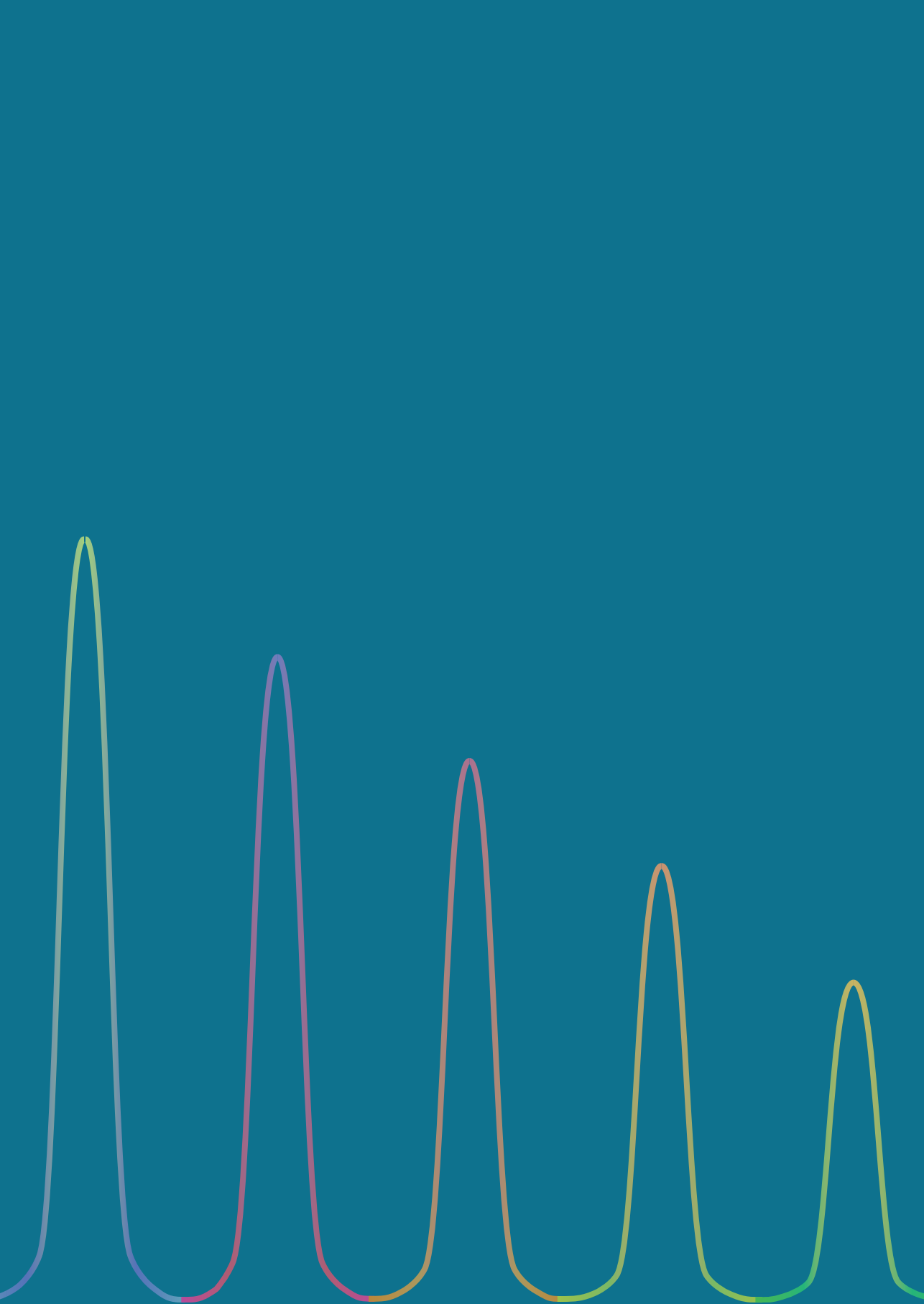
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Chapter 6

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 D₃ (GlcD₃) 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 GlcD₃ (**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), 7 α -hydroxylase (CYP7A1), Cytochrome P450Sc (CYP11A1) or via autooxidation processes (oxidation and epoxidation) [8, 13, 14]. Oxysterols formed by auto-oxidation, like 7-hydroxyperoxycholesterol, 7-ketocholesterol and 7 β -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-3 β ,20 β -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, D₃ is also an substrate for CYP11A1, resulting in the formation of 20-hydroxycholecalciferol (20(OH)D₃) [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 7 α -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 7 α -hydroxycholesterol [15, 39, 40]. 7 α -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 7 α ,8 α -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 D₃, both being

a substrate for CYP27A1. In SLOS patients 7DHC levels are elevated, as well as levels of 25(OH)D₃ [51]. SLOS patients do not show association with osteoporosis, indicating that elevation of 7DHC and 25(OH)D₃ protects against osteoporosis [8, 43, 50]. In CTX patients, 7DHC levels are also elevated [52], while conversion of D₃ into 25(OH)D₃ by CYP27A1 is impaired [53], resulting in lower levels of 25(OH)D₃ [54]. This might explain why SLOS patients 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-glucosylated 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.

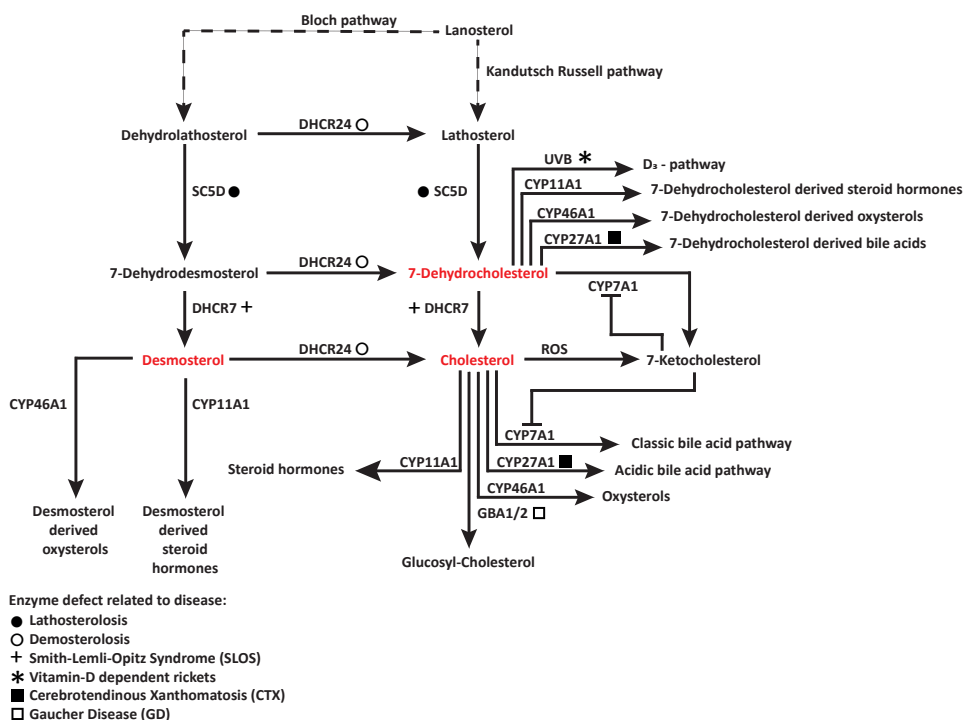


Figure 1. Pathways of desmosterol, 7-dehydrocholesterol and cholesterol related to disease.

Displaying the pathways of desmosterol, 7-dehydrocholesterol and cholesterol. Involved enzymes are Sterol C5-desaturase (SC5D), 7-Dehydrocholesterol Reductase (DHCR7), 24-Dehydrocholesterol Reductase (DHCR24), 7 α -hydroxylase (CYP7A1), Cytochrome P450Scs (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/D₃ 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 D₃ (D₃), 25-hydroxyvitamin D₃ (25(OH)D₃), 1α-25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) 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 D₃ are not measured within Desmosterolosis, but patients show osteosclerosis, which is related to high levels of D₃. Therefore levels are expected to be either elevated or normal. ** As levels of D₃ are unmentioned for Vitamin D- dependent rickets patients, we suspect based on that the enzyme defect is downstream of D₃ 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) ₂ D 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]					+ /N**	+ /N	-
	Type 1B [90-93]					+ /N**	-	N
	Type 2A [90-92, 94-96]					+ /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 GlcD₃. 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
	<i>Lathosterolosis</i> [72-77]	-/N	-/N	-	-
	<i>SLOS</i> [72, 78, 79]	-	+	-	+
	<i>Desmosterolosis</i> [63, 70, 80-86]	-/N	+/N	+	+/N
	<i>CTX</i> [44-46, 48, 49, 52, 54, 87-89]	+/N	+		+/N
<i>Vitamin D - dependent Rickets</i>	<i>Type 1A</i> [90-92]				+/N
	<i>Type 1B</i> [90-93]				+/N
	<i>Type 2A</i> [90-92, 94-96]				+/N
<i>Gaucher Disease</i>	<i>Type I</i> [3, 97-103]	+	N	+	U
	<i>Type II</i> [101]				
	<i>Type III</i> [101, 104]				
<i>Niemann Pick Diseases</i>	<i>Type A</i> [105-107]	+			
	<i>Type C1</i> [3, 108-110]	+	+	+	+
	<i>Type C2</i> [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, GlcD₃ 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).

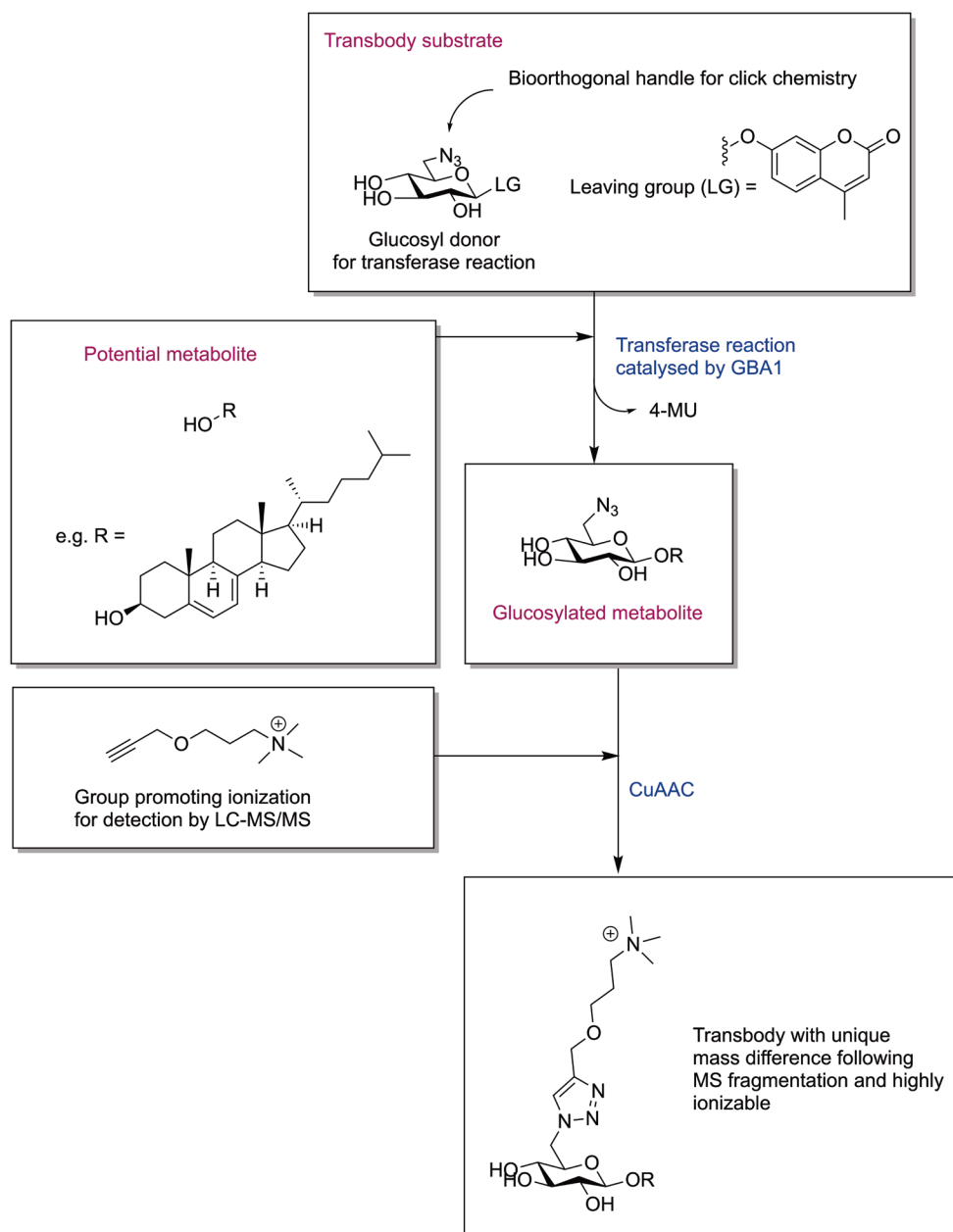


Figure 2. Reverse metabolomics workflow for the discovery of glucose-modified metabolites.

As 'Transbody' substrate a C₆-azide-4MUGlc was synthesized. Incubation with recombinant GBA and selected tissue lysates or selected pure alcohols, are expected to result in novel C₆-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 C₆-azido-GlcChol. With this method we tested the ability of GBA and GBA2 to transglucosylate the given 'Transbody' substrate, C₆-azide-4MUGlc, into C₆-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 C₆ 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, GlcD₃ 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, GlcD₃ 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, GlcD₃ 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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