Role of Lipid rafts in the pathophysiology of lysosomal storage diseases

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List of publications

Maalouf K, von Köckritz-Blickwede M, Das AM, Naim, HY. (2012) M. Niemann Pick C1: Characterization of lipid raft abnormalities and the reversing effects by N-butyl-deoxinojirimycin. *In preparation for submission.*

Maalouf K, von Köckritz-Blickwede M, Das AM, Naim, HY. (2012) M. Anderson-Fabry: Abnormalities of lipid rafts can be reversed by enzyme replacement or substrate reduction. *Submitted to Orphanet Journal of Rare Diseases*.

Maalouf K, Jia J, Rizk S, Brogden G, Keiser M, Das A, Naim HY. (2010) A modified lipid composition in Fabry disease leads to an intracellular block of the detergent-resistant membrane-associated dipeptidyl peptidase IV. *J of Inherit Metab Dis.* 33:445-9.

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Maalouf K, Amiri M, Das AM, Naim HY M. niemann-pick type c: restoration of lipid rafts and other biochemical anomalies by n-butyl-deoxinojirimycin. 4th Scientific Symposium on Niemann-Pick Type C: *The Expanding Univse of NP-C***.** 24–25 March 2012, Athens, Greece (Poster).

Maalouf K, Naim HY, Das AM. M. Fabry: Abnormalities of lipid-rafts can be reversed by ERT. *APS-working group for pediatric metabolic disorders annual meeting,* 8th March 2012, Fulda, Germany (Poster).

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Maalouf K, Das AM, Naim HY. M. niemann-pick type c: restoration of lipid rafts and other biochemical anomalies by n-butyl-deoxinojirimycin. *SSIEM annual symposium 2011, International Conference Centre Geneva (CICG)*, August 30th-September 2nd, Geneva, Switzerland (Poster).

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Maalouf K, Das AM, Naim HY. A modified lipid composition in Fabry's disease leads to an altered trafficking of certain lipid raft-associated proteins. *APS-working group for pediatric metabolic disorders annual meeting*, 11th March 2011, Fulda, Germany (Talk).

Maalouf K, Brogden G, Jia J, Rizk S, Keiser M, Das AM, Naim HY. Lipid Composition Modifications in Fabry's Disease and their Effect on Detergent-Resistant Membranes. *1st International Symposium for PhD Students on Protein Trafficking in Health and Disease.* May 26th-28th 2010, Hamburg, Germany (Poster).

Zusammenfassung

Die lysosomale Speicherkrankheit (LSK) gehoert zu einer Gruppe von vererbbaren Stoffwechselerkrankungen, die durch lysosomale Enzyme oder Proteine verursacht werden und moeglicherweise zu einer verstaerkten Akkumulation, von nicht verstoffwechselten Substraten oder falsch lokalisierten Proteinen fuehren. Bisher wurden viele LSK-assoziierte Proteine und Speichermolekuele identifiziert. Allerdings sind die genauen Signaltransduktionswege, die LSK beeinflussen noch unzureichend geklärt. Ziel dieser Studie war es die biochemischen Signalwege zu untersuchen, die einen Einfluss auf die Pathogenese von Morbus Fabry und Niemann Pick Typ C1 haben. In dieser Doktorarbeit konnte gezeigt werden, dass bei beiden Krankheiten die Membranzusammensetzung und Membranfunktion durch eine Anreicherung von Glykolipiden und Cholesterol beeintraechtigt wird, wodurch weitere Signaltransduktionswege wie Trafficking- und Endozytose-Mechansimen veraendert werden. Darueber hinaus konnte in dieser Arbeit gezeigt werden, dass “Lipid Raft” von Morbus Fabry Zellen und NPC1 Zellen moduliert werden. Bei NPC1 Zellen akkummulierte die “Lipid raft”-assoziierte Dipeptidylpeptidase IV (DPPIV) durch eine verlangsamte Endozytose auf der Membranoberflaeche. Bei Morbus Fabry konnte eine intrazellulaere Akkumulation von DPPIV gezeigt werden. “Lipid rafts” spielen also in der Pathogenese von Glykolipid/Cholesterol-LSK eine zentrale Rolle. Diese Ergebnisse ermoeglichen sowohl ein besseres Verstaendnis als auch die Identifizierung von neuen Biomarkern bei LSK.

Stichwoerter: lysosomale Speicherkrankheit, “Lipid rafts”, Niemann Pick TypC1, Morbus Fabry, Pathogenese

Abstract

Lysosomal storage diseases (LSDs) are a group of inherited metabolic disorders caused by a defect in a lysosomal protein/enzyme, which eventually leads to a progressive lysosomal accumulation of the involved unmetabolised substrate or untrafficked molecule. To date many of the defective proteins and their relative accumulating compounds have been identified. However, when it comes to secondary biochemical pathways involved in the pathogenesis of most LSDs, the literature is still prominently lacking. The aim of this study was to investigate biochemical pathways involved in the pathogenesis of two major lipid storage diseases: M. Fabry (a glycolipid storage disorder) and Niemann Pick type C1 (NPC1, a cholesterol-glycolipid storage disease). A variety of tests revealed that the membrane composition and function in particular that of microdomains which are enriched in glycolipids and cholesterol is impaired in both diseases, subsequently leading to an impairment of other related biochemical pathways and events such as endocytosis and protein trafficking. Lipid rafts marker protein flotillin 2 showed an altered distribution in Triton X-100 detergent resistant membranes (DRMs) from Fabry and NPC1 cells. Trafficking of dipeptidyl peptidase IV (DPPIV) -a lipid rafts associated protein- was intracellularly blocked in Fabry cells, whereas in NPC1 cells DPPIV accumulated on the cell surface due to a delay in endocytosis. Thus, lipid rafts seem to play a key role in pathogenesis of glycolipid/cholesterol storage diseases. These results help in a better understanding of pathogenesis of such disorders and pave the way for the possibility to identify new biomarkers along the various pathways implicated with lipid rafts.

Keywords: Lipid rafts, Lysosomal storage diseases, Fabry, Nieman-Pick C, pathogenesis

Abbreviations list

TGN	Trans-Golgi network
MPR	Mannose-6-phosphate receptor
LE	Late endosome
AGAL	Alpha-galactosidase
LSD	Lysosomal storage disease
NPC	Niemann-Pick type C
MPS	Mucopolysaccharidoses
CNS	Central nervous system
ERT	Enzyme replacement therapy
SRT	Substrate reduction therapy
BBB	Blood brain barrier
DRM	Detergent resistant membranes
SM	Sphingomyelin
Gb3	Globotriaosylceramide
DPPIV	Dipeptidyl peptidase IV
APN	Aminopeptidase N

GSL	Glycosphingolipids
GD	Gaucher disease
TX-100	Triton-X-100
GC	Glucosylceramide
SLSD	Sphingolipid storage diseases
WT	Wild type

CHAPTER 1

Introduction

1. Introduction

When one of the lysosomal proteins/enzymes is defective, it causes an accumulation of the relative unmetabolized substrate inside the lysosomes. This phenomenon represents the general characteristic of lysosomal storage diseases (LSDs), a family of rare inherited metabolic disorders. The progress of pathogenesis in LSDs is still not very well understood. One possible mechanism postulate that alterations in “lipid raft” occur in lipid storage disorders and contribute in their pathogenesis. Lipid rafts are cholesterol-sphingolipid enriched microdomains within the cell membrane.

1.1 Lysosomes

The discovery of the lysosomes (previously labeled as suicide bags) in 1949 by Christian de Duve paved the way for a better understanding of these organelles and their associated activities. Lysosomes constitute the primary compartments responsible for the majority of catabolic reactions which take place in eukaryotic cells. They contain enzymes that will degrade various polymers including carbohydrates, proteins, nucleic acids and lipids that reach the lysosomes through endocytosis, phagocytosis or autophagy. These enzymes are collectively termed *acid hydrolases* as they function best at a pH ≈ 4.8 which is the lysosomal internal pH maintained by a proton pump and a Cl^- channel protein present in the lysosomal membrane (1). Lysosomes are present in various shapes and sizes and constitute up to 5% of the intracellular volume (2). The function of lysosomes is not strictly associated with degradation of various substrates and pathogens, they aid in plasma membrane repair during cell injury, play a role in coordinating intracellular signaling and metabolic activities and they have more specialized secretory functions in some cell types (2, 3)

1.1.1 Classification of lysosomal proteins

Lysosomal proteins can be classified into two major groups: soluble lysosomal hydrolases (otherwise known as acid hydrolases) and lysosomal membrane proteins (LMPs). The two classes are thought to have distinct biogenesis pathways and functions. More than 50 types of acid hydrolases are involved in degrading a variety of specific substrates, antigen processing, extracellular matrix degradation and apoptosis. Acid hydrolases are in turn classified into: lipases, glycosidases, proteases, nucleases, phosphatases and sulfatases, with the name of each class indicative of its function. Alpha-galactosidase A (AGAL) which will be addressed thoroughly in this study is an example of a lipase responsible for biodegradation of glycosphingolipids. LMPs like NPC1 transporter protein (the other major focus of this study) have several functions including: acidification of the lysosomal lumen, protein import from the cytosol, membrane fusion and transport of degradation products to the cytoplasm (4).

1.1.2 Biogenesis of lysosomal proteins

Newly synthesized lysosomal proteins are targeted to lysosomes either by following a constitutive secretory pathway to the plasma membrane and eventually reach the lysosomes via endocytosis, or by a direct intracellular pathway via mannose-6-phosphate residues (Fig1). Lysosomal proteins following the direct intracellular pathway get phosphorylated in the cis Golgi network, after which they bind mannose-6-phosphate receptors (MPRs) in the trans-Golgi network (TGN). From the TGN they are transferred via clathrin coated vesicles to late endosomes (LEs) where they will dissociate from their MPRs due to the acidic pH and get dephosphorylated. Free hydrolases are then delivered to lysosomes and the MPRs are recycled back to TGN or cell membrane (1, 2, 4). Not too much is known about LMPs, but the literature

suggests that unlike soluble hydrolases, they do not necessitate MPRs. They are targeted from the TGN to lysosomes via a direct route which involves adapter proteins or an indirect route through the plasma membrane (2).

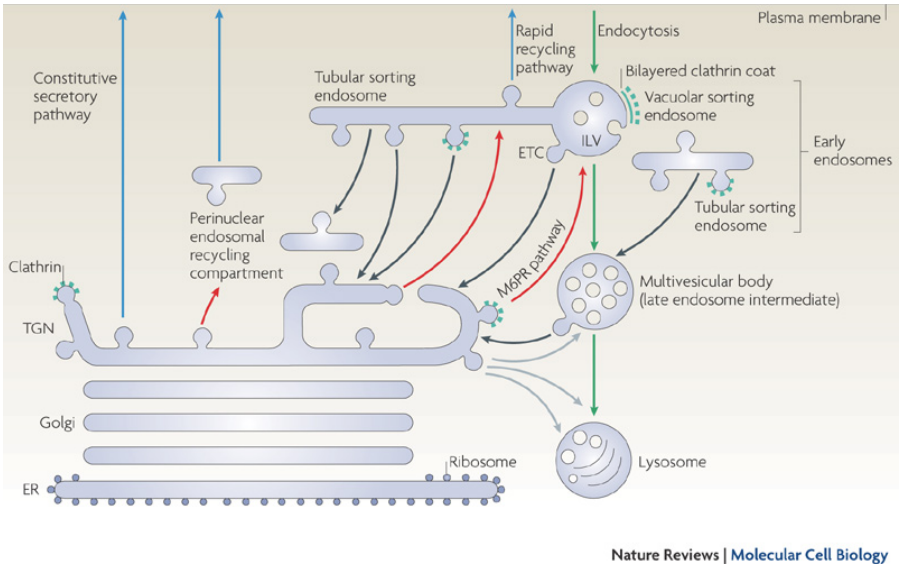

Figure 1 Newly synthesized lysosomal proteins follow from the TGN a constitutive secretory pathway (blue arrows) to the plasma membrane and eventually reach the lysosomes by endocytosis (green arrows), or they follow a direct intracellular pathway (e.g. via M6PRs) to the endo-lysosomal system (red arrows) (4).

1.2 Lysosomal Storage Diseases (LSDs)

When one of the acid hydrolases or the LMPs is defective or missing, it results in deficient processing of the substrate which in turn leads to accumulation of the relevant substrate within the lysosome and ultimately within the cell. This phenomenon describes a family of diseases known as the lysosomal storage diseases (LSDs). There are more than 40 types of LSDs involving acid hydrolases and a fewer number involving LMPs described so far, each resulting from a specific genetic defect.

1.2.1 Classification of LSDs

The classification of LSDs had been subject to some controversy. Initially LSDs were characterised based on the type of substrate being accumulated. For instance, LSDs such as Fabry, Gaucher, and Tay-Sachs which accumulate globotriasylceramide, glucosylceramide, and GM2 ganglioside respectively would be grouped under sphingolipidoses category. This class of LSDs accumulates mainly sphingolipids and consists of at least nine disorders characterized by a wide range of clinical manifestations involving liver, spleen, kidneys, bone, and CNS (5). Other LSDs which accumulate mucopolysaccharides are known as mucopolysaccharidoses (MPS). However, there are some like Futerman and van Meer (6) who argue that the most useful method to classify LSDs is the one based on the defective protein or enzyme, since with the previously mentioned method there were many erroneous disease characterizations which occurred because the discovery of many accumulating substrates happened before their relative defective enzyme was identified. For example, Niemann-Pick C (NPC) was initially characterized as a sphingomyelin disorder along with Niemann-Pick A and B which are caused by a defect in sphingomyelinase activity. It is now evident that NPC is caused by a mutation in transporter proteins NPC1 and NPC2 (6).

1.2.2 Therapy of LSDs

Even though different LSDs encompass distinctive types of storage materials, nonetheless they do share several common biochemical, cellular and clinical traits. Most LSDs are monogenic and the majority of patients accumulate storage material in both viscera and the central nervous system (CNS). Therefore therapy measures as summarized in figure 2 are similar for most LSDs. Originally the treatment for most LSDs was symptomatic, so patients

were basically given painkillers and were monitored for kidney, liver, spleen and other complications. In 1991 a study were enzyme replacement therapy (ERT) was applied for the first time was reported by Barton et al. 1992 (7), it involved the infusion of human placental glucocerebrosidase into 12 patients with type 1 Gaucher disease. Plasma glucocerebrosidase levels decreased in 9 patients out of 12, and all patients were reported to have decreased splenic and hepatic volumes after six and five months of treatment, respectively. ERT for other LSDs such as Fabry and Mucopolysaccharidoses and Pompe disease followed later on. One major limitation in ERT is the inability of infused enzymes to penetrate the blood brain barrier (BBB) and thus making a positive response for the CNS manifestations not possible, another issue is that the infused enzyme may lead to developing antibody against it, in addition to the high cost for producing recombinant enzymes through biotechnology which makes this therapy a relatively expensive one. Substrate reduction therapy (SRT) is being now more considered as a therapeutic option for most LSDs. The interest in SRT developed after the discovery of certain small molecules especially imino sugars that inhibit the synthesis of accumulating substrates and at the same time are able to cross the BBB. Even more attention was given to such molecules after it was known that they also act as chaperones and thus enhance the functionality of defective enzymes correcting misfolded proteins and hence leading to their proper lysosomal targeting. N-butyldeoxynojirimycin (NB-DNJ) (OGT 918, miglustat, Zavesca®manufactured by Actelion) inhibits glucosylceramide-synthetase. This substance is now commercially available for the treatment of two LSDs, M. Gaucher and M. Niemann-Pick type C (NPC). Gene therapy and stem cell transplantation are other therapeutic options that have been considered for the treatment of LSDs. However, these procedures still face many

associated risks of graft failure or transcriptional interference by vectors, hence there's still a long way before they can become realistic therapeutic options for LSDs.

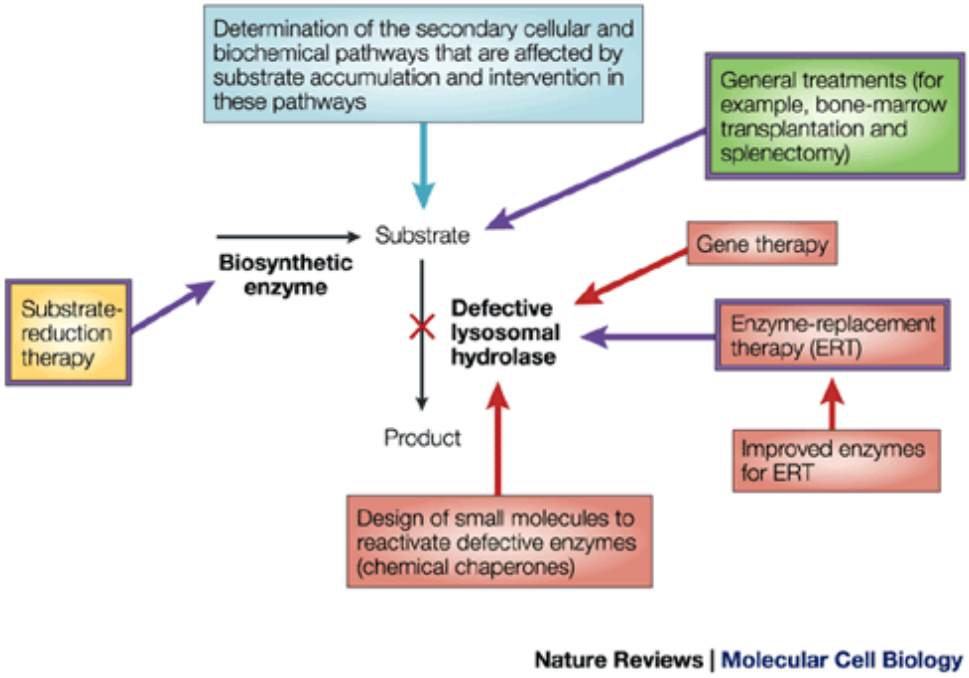

Figure 2 Therapeutic options for LSDs (6)

1.2.3 Pathophysiology of LSDs

Along the past years a great progress has been made in describing many of the genetic, enzymatic and molecular bases of numerous LSDs. Yet the events which occur after substrate accumulation that lead to cell dysfunction in LSDs are not yet widely understood. However, several downstream pathways have been described to be implicated in the pathology of LSDs. It was proposed that there is a compromised lysosomal stability or integrity in LSD cells which may lead to discharge of some hydrolases or accumulating substrates into the cytosol, and that the lysosomal storage capacity overload would eventually lead to a defective intracellular trafficking of compounds from and to the lysosomes (6). Other described pathogenic

downstream pathways implicated in LSDs include altered calcium homeostasis, oxidative stress, inflammation, altered lipid trafficking, autophagy, endoplasmic reticulum stress, and autoimmune responses (8). Based on the changes in lipid cell composition present in most LSDs, we hypothesized that “lipid rafts” or what is known as detergent resistant membranes (DRMs) may play an additional role in LSD pathogenesis.

1.3 Biological membrane and lipid rafts

Cell membranes contain a large number of lipids and various proteins. Eukaryotic membrane lipids are glycerophospholipids, sphingolipids, and sterols. The apmphipathic nature of lipids allows them to interact with other lipids through their hydrophobic domains and with water through their polar heads (9). When Singer and Nicolson tried to describe the structural feature of the animal cell membrane forty years ago, they proposed the so called “fluid mosaic model”. According to this model, the plasma membrane consisted of a lipid bilayer through which various membrane proteins are integrated arbitrarily (10). This rather simplified model was replaced in later years by a more complex one after the concept of membrane microdomains which traverse the homogenous fluid lipid bilayer emerged.

1.3.1 Classification of membrane domains

Membrane domains could be protein based or lipid based. Protein based domains can be arranged into small membrane domains which arise from direct interactions, or large protein domains and these require various protein scaffolds to be formed (11). Lipid based domains are membrane regions enriched in cholesterol and sphingolipids along with a collection of membrane proteins (11). This clustering occurs mainly due to the tendency of sterol groups to pack tightly with saturated acyl chains of lipids, and it results in the segregation of the lipid

bilayer into a liquid-ordered (Lo) phase enriched in cholesterol and glycosphingolipids and a liquid-disordered phase (Ld) consisting mainly of phospholipids and other glycerophospholipids (Figure 3) (12). Lipid microdomains which constitute the Lo phase are highly condensed and have the ability to resist solubilization by certain nonionic detergents such as Triton X-100 (TX-100) at cold temperatures, and hence they are referred to as detergent resistant membranes (DRMs). These Lo phase microdomains are referred to as the "lipid rafts" and they function as platforms within which various proteins are embedded, such as glycosylphosphatidylinositol (GPI)-anchored proteins, transmembrane proteins (often palmitoylated) and di-acylated proteins (13).

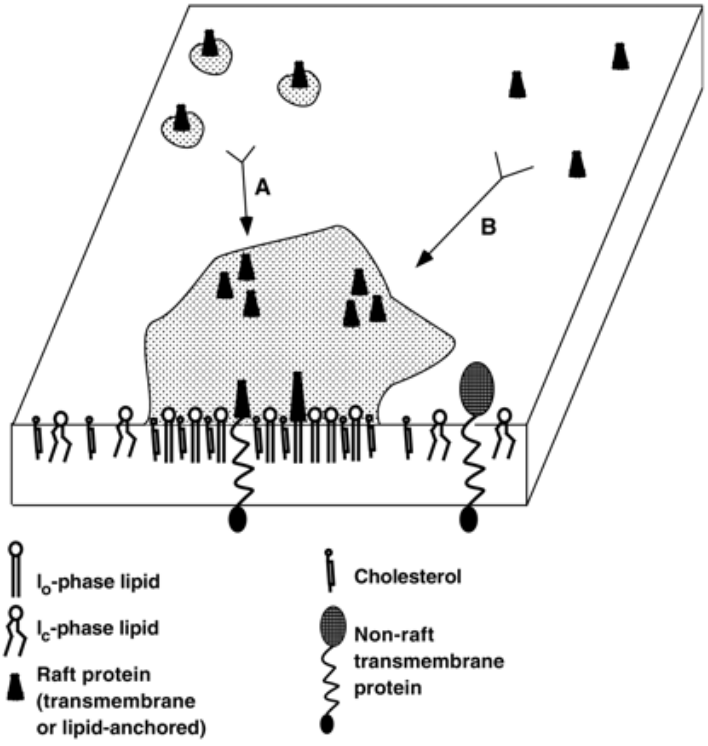

Figure 3 This model shows how tightly packed cholesterol and sphingolipids organize into Lo phase rafts (light gray), and how raft proteins could be recruited as small raft clusters (A) or as individual proteins (B) into the larger rafts (14).

Not much is known about the size, shape, stability, and composition of these membrane rafts. Contradictory models have been proposed which distinguish between steady state short-lived platforms or “reserve rafts” that are small, unstable and exist in resting cells, and long-lived “receptor-cluster rafts” that are large, stabilized rafts that form after stimulation or cross-linking of raft molecules (15). However, long-lived mobile platforms were also observed in resting cells by using a new direct imaging methodology based on single molecule microscopy and photobleaching approach (16).

1.3.2 Diversity of lipid rafts composition and function

Different organelles have specific lipid compositions, which are important in determining their shape and functions. The possibility to extract DRMs with various mild detergents such as TX-100, Lubrol WX, Brij 58 and Tween 20 permits the characterization of different types of microdomains. For instance, Triton X-100 DRMs contain cholesterol/sphingolpid-enriched structures in the trans-Golgi network. The endoplasmic reticulum (ER) has a different membrane lipid composition of phospholipid clusters, such as phosphatidylcholine and phosphatidylethanolamine which can be extracted using Tween 20 or Brij 58. Lubrol-DRMs are low in cholesterol and sphingolipids and usually contain mature Golgi processed proteins (11). This variation in lipid composition of different organelle membranes may indicate also a variation in the size and function of each type of raft. Sorting of proteins and lipids along the secretory pathway seems to be directed by lipid rafts. For instance, apically sorted proteins in epithelial cells were found to associate with Tween 20 DRMs, whereas basolateral proteins were completely solubilized by this detergent, indicating an early polarized sorting which starts at the level of ER (17).

Cell signaling, intracellular membrane transport, cell adhesion and host-pathogen interactions are among the cell processes regulated by lipid rafts (18, 14). Some raft types such as caveolae appear to be involved in endocytosis. These structures contain cholesterol-binding protein caveolin and they tend to form cell surface invaginations and are eventually internalized (13).

Aim of this study

The aim of this study was to investigate the role of lipid rafts in pathogenesis of lysosomal storage diseases. The implications of lipid rafts were addressed in two LSDs, M. Fabry **[Chapter 2, Chapter 3]** and M. Neimann Pick type C1 disease **[Chapter 4]**.

The condensed state of glycolipid/cholesterol enriched microdomains renders them resistant to solubilization by non-ionic detergents such as Triton X-100.

To check for membrane composition alterations in fibroblasts from Fabry patients, lipid analysis of Triton X-100 DRMs was performed. Trafficking of lipid raft associated protein dipeptidylpeptidase IV (DPPIV) was examined in the same Fabry cells **[Chapter 2]**. To confirm any lipid raft alteration in Fabry cells, distribution of lipid rafts marker protein flotillin 2 was investigated. ERT/SRT was then applied to Fabry cells to check whether restoration of glycolipid content in these cells would rescue any identified biochemical anomaly **[Chapter 3]**.

In fibroblasts from NPC1 patients, the distribution of lipid raft marker protein flotillin 2 was examined. In parallel, the trafficking of DPPIV to the membrane was tested by doing cell surface protein isolation and FACS analysis. To further comprehend what mechanism is responsible for the accumulation of lipid raft associated protein DPPIV on the cells surface of NPC1 cells, an endocytosis assay was conducted to compare the internalization rate of DPPIV in NPC1 fibroblasts relative to normal fibroblasts **[Chapter 4]**.

In **Chapter 5** the various findings of this study are discussed and analyzed concluding that lipid rafts are indeed a major contributing factor to the pathogenesis of LSDs.

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Chapter 2

A modified lipid composition in Fabry disease leads to an intracellular block of detergent-resistant membrane-associated dipeptidyl peptidase IV

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http://www.springerlink.com/content/v6022657002637r7/

Katia Maalouf's contributions to this article are the following:

- Design of individual experiments
- Performing experimental part of the study: (Figure 2,B &C) (Figure 3, B)
- Analysis and interpretation of the results
- Writing answers for the reviewers

Abstract

Fabry disease is an X-linked lysosomal storage disorder that leads to abnormal accumulation of glycosphingolipids due to a deficiency of alpha-galactosidase A (AGAL). The consequences of these alterations on the targeting of membrane proteins are poorly understood. Glycosphingolipids are enriched in Triton-X-100- resistant lipid rafts [detergent-resistant membranes (DRMs)] and play an important role in the transport of several membrane-associated proteins. Here, we show that in fibroblasts of patients suffering from Fabry disease, the colocalization of AGAL with the lysosomal marker LAMP2 is decreased compared with wild-type fibroblasts concomitant with a reduced transport of AGAL to lysosomes. Furthermore, overall composition of membrane lipids in the patients' fibroblasts as well as in DRMs reveals a substantial increase in the concentration of glycolipids and a slight reduction of phosphatidylethanolamine (PE). The altered glycolipid composition in Fabry fibroblasts is associated with an intracellular accumulation and impaired trafficking of the Triton-X-100 DRM-associated membrane glycoprotein dipeptidyl peptidase IV (DPPIV) in transfected Fabry cells, whereas no effect could be observed on the targeting of aminopeptidase N (ApN) that is not associated with this type of DRM. We propose that changes in the lipid composition of cell membranes in Fabry disease disturb the ordered Triton X-100 DRMs and have implications on the trafficking and sorting of DRM-associated proteins and the overall protein–lipid interaction at the cell membrane. Possible consequences could be altered signaling at the cell surface triggered by DRM-associated proteins, with implications on gene regulation and subsequent protein expression.

Abbreviations: AGAL, alpha-galactosidase A; ApN, aminopeptidase N; DPPIV dipetidylpeptidase IV; DRM, detergent-resistant membranes; GFP, green fluorescence protein; PC, phosphatidylcholine; PE Phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine

Competing interest: None declared

Katia Maalouf and Jia Jia contributed equally

Introduction

Fabry disease (Anderson-Fabry disease) is an X-linked lysosomal storage disorder caused by deficiency of α-galactosidase A (AGAL, gene symbol *GLA*, NM_0001692) (1) and results in abnormal accumulation of glycosphingolipids that are associated with several clinical signs and symptoms. So far, more than 400 mutations have been described in the coding sequence of AGAL (2), many of which are specific for one family (3). Recent studies showed that cellular lipid trafficking and processing are disturbed in lysosomal storage diseases, such as Fabry disease (4, 5). We therefore assumed that alterations in lipid compositions would lead to disturbances in the ordered structure of a particular type of membranes, detergent-resistant membranes (DRMs) and /or lipid rafts, which are enriched in glycosphingolipids and cholesterol. Given that many proteins are transported or targeted to their final cellular destinations via DRMs, it is plausible to assume that alterations in the DRM composition could affect the trafficking of DRM-associated proteins in Fabry fibroblasts. The aim of this study was to investigate the transport of dipeptidyl peptidase IV (DPPIV) and aminopeptidase N (ApN) in fibroblasts of patients suffering from Fabry disease and of a healthy individual. Both proteins are transported

to the cell surface via association with different types of lipid rafts or DRMs. DPPIV associates in the trans-Golgi network with Triton-X-100-resistant cholesterol/sphingolipids-enriched DRMs (6). ApN is transported to the cell surface independently of Triton X-100 DRMs but associates prior to the cis-Golgi with Tween20 DRMs that are enriched in phosphatidylcholine and phosphatidylethanolamine and devoid of sphingolipids (7).

Material and methods

Cell culture and substances

Fibroblasts from patients with Fabry disease and a healthy person were grown in Dulbecco's modified Eagle's medium (DMEM) (PAA, Coelbe, Germany) supplemented with 10% fetal bovine serum (BioWest, Nuaillé, France), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C, 95% humidity, and 5% carbon dioxide (CO_2). All other chemicals were obtained from Sigma, Taufkirchen, Germany, and were of analytical grade.

Identification of a novel mutation in GLA

The full-length *GLA* was amplified using reverse-transcribed RNA from fibroblasts (indicated U5) of diseased patients (forward primer: 5'-CAATGCAGCTGAGGAACCCAG-3', reverse primer: 5'-CTAAAATTTGAGGTTGTTACT-3'). Polymerase chain reaction (PCR) products were cleaned and used for direct sequencing by Eurofins MWG Operon, Ebersberg, Germany.

Transfection and subcellular localization of proteins by confocal laser microscopy

Fabry and control fibroblasts were transiently transfected with complementary DNA (cDNA) clones encoding chimeric forms of ApN and DPPIV tagged with the green fluorescence protein (GFP), i.e. ApN-GFP and DPPIV-GFP (8). The transfection reagents used were FuGENE HD

(Roche, Mannheim, Germany) or Nanofectine (PAA, Pasching, Austria). For indirect immunofluorescence of AGAL, the protein was stained using the polyclonal rabbit α-gal A (H-104) antibody (Santa Cruz Biotechnology, Heidelberg, Germany), followed by an anti-rabbit secondary antibody conjugated with Alexa Fluor 568. The lysosomal marker LAMP2 was immunolabeled using the polyclonal mouse antibody LAMP-2 (H4B4) (Santa Cruz Biotechnology), followed by an anti-mouse secondary antibody conjugated to Alexa Fluor 488. Confocal laser microscopy was performed in a Leica TCS SP2 or SP5 microscope (Leica Microsystems, Bensheim, Germany). The ratio of AGAL and LAMP2 colocalization was calculated using ImageJ software (NIH, Bethesda, Maryland).

Quantification of lipid composition and glycolipid analysis

Lipids were isolated by the method of Bligh and Dyer (9) and analyzed by high-performance liquid chromatography (HPLC). The glycolipids were separated by high-performance thin-layer chromatography (HPTLC), followed by staining with 2 μg/ml orcinol in 20% sulfuric acid (H2SO4), where equal amounts from each sample were loaded on the TLC plates. Student's *t* test was performed to determine statistical significance. Differences were considered significant at $P < 0.05$.

Results and discussion

In fibroblasts (indicated U5) of a patient suffering from Fabry disease, we identified a new mutation in exon two of the coding sequence of AGAL (c320A>T), resulting in the amino acid exchange glutamine to leucine at residue 107 (Q107L). These fibroblasts and fibroblasts of a healthy individual, referred to hereafter as wild-type fibroblasts, were used to study the subcellular localization of AGAL. For this study, cells were colabeled with antibodies against

human AGAL and the lysosomal protein marker LAMP2. By indirect immunofluorescence microscopy, we could detect alterations in the level of colocalization of LAMP2 with AGAL in wild-type and Fabry fibroblasts (Fig1). Thus, the level of colocalization of LAMP2 and AGAL was reduced substantially from >95% in wild-type cells to approximately 72% in Fabry fibroblasts. This indicates that the amino acid exchange leads to partial trafficking of AGAL to the lysosomes, eliciting symptoms of Fabry disease.

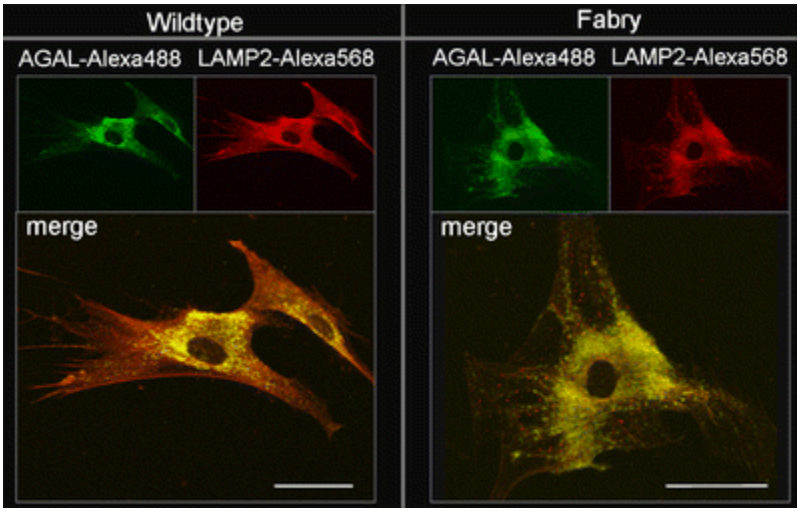

Figure 1 Subcellular localization of alpha-galactosidase A (AGAL) and the lysosomal protein marker LAMP2 in wild-type and Fabry fibroblasts. AGAL was stained using the polyclonal rabbit α-gal A antibody, followed by an anti-rabbit secondary antibody conjugated with Alexa Fluor 568. The lysosomal marker LAMP2 was immunolabeled using the polyclonal mouse antibody LAMP-2, followed by an anti-mouse secondary antibody conjugated to Alexa Fluor 488. Confocal laser microscopy was performed in a Leica TCS SP2 microscope. The ratio of AGAL and LAMP2 colocalization was calculated with ImageJ. AGAL is labelled in *green*, LAMP2 in *red*, and colocalization in *yellow*. *Scale bars* 20 µM.

Analysis of the lipid composition of wild-type and Fabry fibroblasts showed an approximately threefold increase in the concentration of glycolipids and a slight reduction in the concentration of phosphatidylethanolamine (PE) of about 5% (Fig2a). These findings were statistically relevant ($P = 002$ and $P = 003$, respectively). Analyses of phosphatidylinositol (PI),

phosphatidylserine (PS), and phosphatidylcholine (PC) did not yield statistically relevant changes. Also,analysis of lipid composition in DRMs revealed an elevated level of sphingomyelin, whereas the levels of phospholipids were almost similar to control cells (Fig2b). Measurements were also performed with another Fabry cell line (U2) to determine whether these changes represent a general Fabry phenotype. Here again, elevated levels of sphingomyelin were demonstrated in line with the view that the general pattern of glycolipids is altered. To determine the changes in glycolipid composition, total membrane lipids were isolated and analyzed by HPTLC. Four glycolipids were recognized at measurable concentrations. The glycolipids were labelled GL1, GL2, GL3, and GL4 and were identified by their respective Rf values of 0.040, 0.17, 0.77, and 0.87 (Fig2c). The results show that the overall glycolipid content increases significantly between the two Fabry cell lines and the control, particularly GL1 and GL4, where GL1 showed an approximately fourfold and fivefold increase in U2 and U5, respectively. GL4 showed a fourfold increase in U5 only. GL2 and GL3 revealed no significant changes.

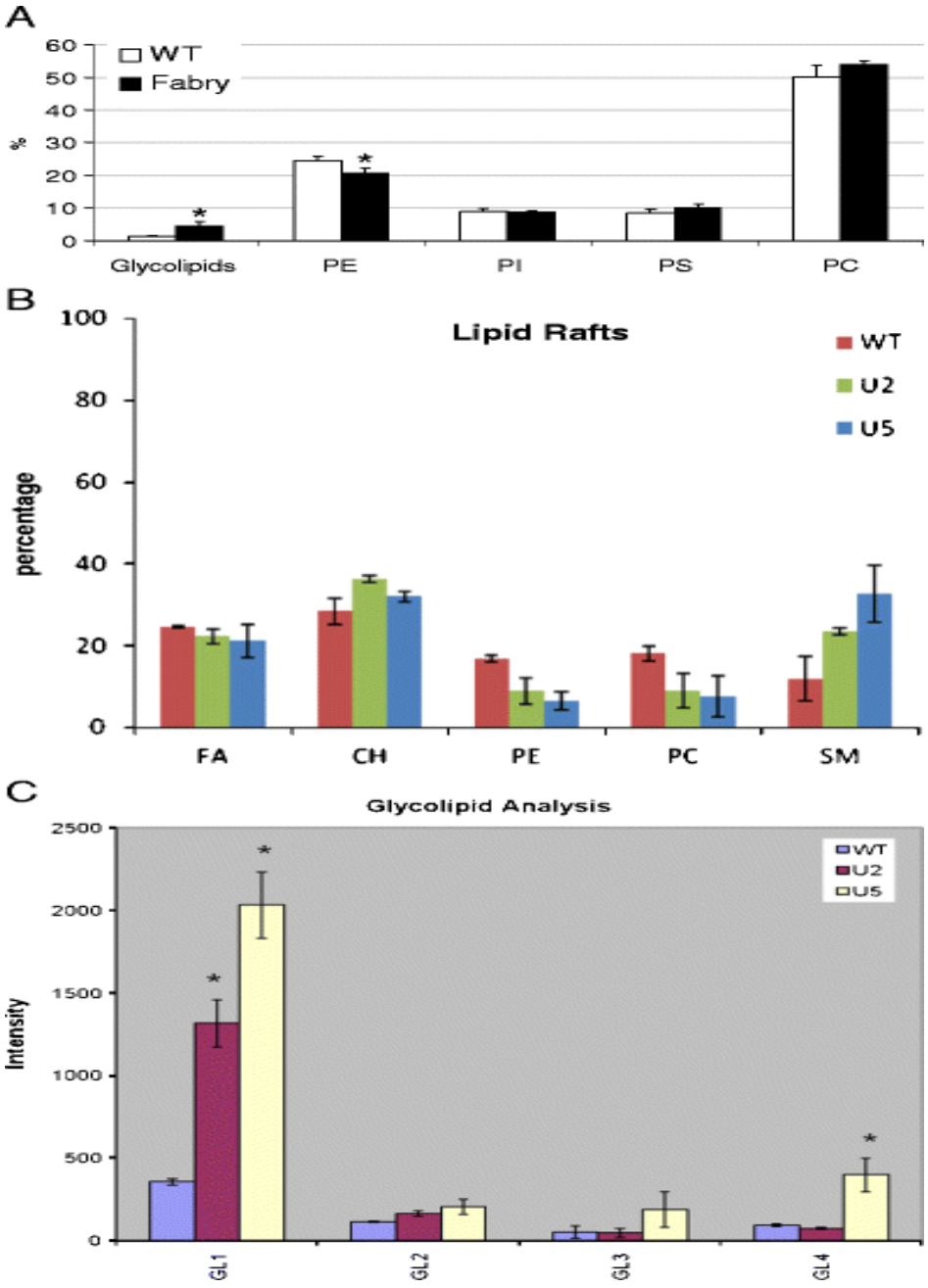

Figure 2 Membrane lipid analysis of wild-type and Fabry fibroblasts. A) Lipid analysis by high-performance liquid chromatography (HPLC): Wild-type (WT) lipids are depicted in *white columns* and Fabry lipids in *black columns*. The proportion of each lipid is given in percent. B) Triton X-100 lipid-raft analysis by high-performance thin-layer chromatography (HPTLC). WT lipids were compared with Fabry lipids (U2 and U5). The proportion of each lipid is given in percent. C) Glycolipid analysis by HPTLC and orcinol staining. The results are shown in terms of band intensity. Statistical analysis employed Student's *t* test, and differences at $P < 0.05$ were considered significant. Data are shown as mean ±

standard deviation (SD) of at least three experiments (*$P < 0.05$ for comparison with WT cells). *FA* fatty acids, *CH* cholesterol, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *PC* phosphatidylcholine, *SM* sphingomyelin.

The substantial increase of glycolipids in patients' fibroblasts could be due to intracellular accumulation and suggests a misbalanced composition of cellular membranes. Many proteins are transported to the cell surface in association with cholesterol/glycolipids-enriched DRMs. In view of this, we examined the trafficking of DRM-associated and nonassociated proteins to the plasma membrane. DPPIV is a type II ubiquitous membrane glycoprotein that is expressed in a variety of polarized and nonpolarized cells and is sorted to the apical membrane via association with DRMs in the trans-Golgi network (6). ApN, on the other hand, is a representative of proteins that are not associated with glycosphingolipid-enriched DRMs. These two proteins were conjugated to the GFP and transiently expressed in wild-type fibroblasts and fibroblasts of a patient with Fabry disease indicated as U5 fibroblasts. Figure 3a shows that DPPIV-GFP and ApN-GFP are predominantly localized at the cell surface in the wild-type fibroblasts (upper panels). By contrast, a strong intracellular and vesicular staining of DPPIV-GFP was observed in the U5 Fabry fibroblasts concomitant with an accumulation of this protein intracellularly and consequently defective trafficking to the cell surface (Fig3a, lower panel/right). On the other hand, the cellular localization of ApN-GFP in U5 Fabry fibroblasts did not change markedly in comparison with its counterpart in wild-type fibroblasts compatible with normal trafficking of this protein (Fig3a, lower panel/left). To determine whether this trafficking behavior is a general phenomenon in Fabry cells, we examined the trafficking of DPPIV and ApN in another Fabry cell line indicated as U2 fibroblasts. Figure 3b demonstrates the strong intracellular staining of DPPIV-GFP in U2 fibroblasts, as well as partial staining at the cell surface. Here again,

the wild-type fibroblasts revealed predominant cell-surface expression of DPPIV-GFP. ApN-GFP was mainly found at the cell surface in wild-type and U2 Fabry fibroblasts. Results support the previous data obtained with the U5 cell line and the notion that the trafficking pattern of the DRM-associated protein DPPIV-GFP is altered in Fabry cells.

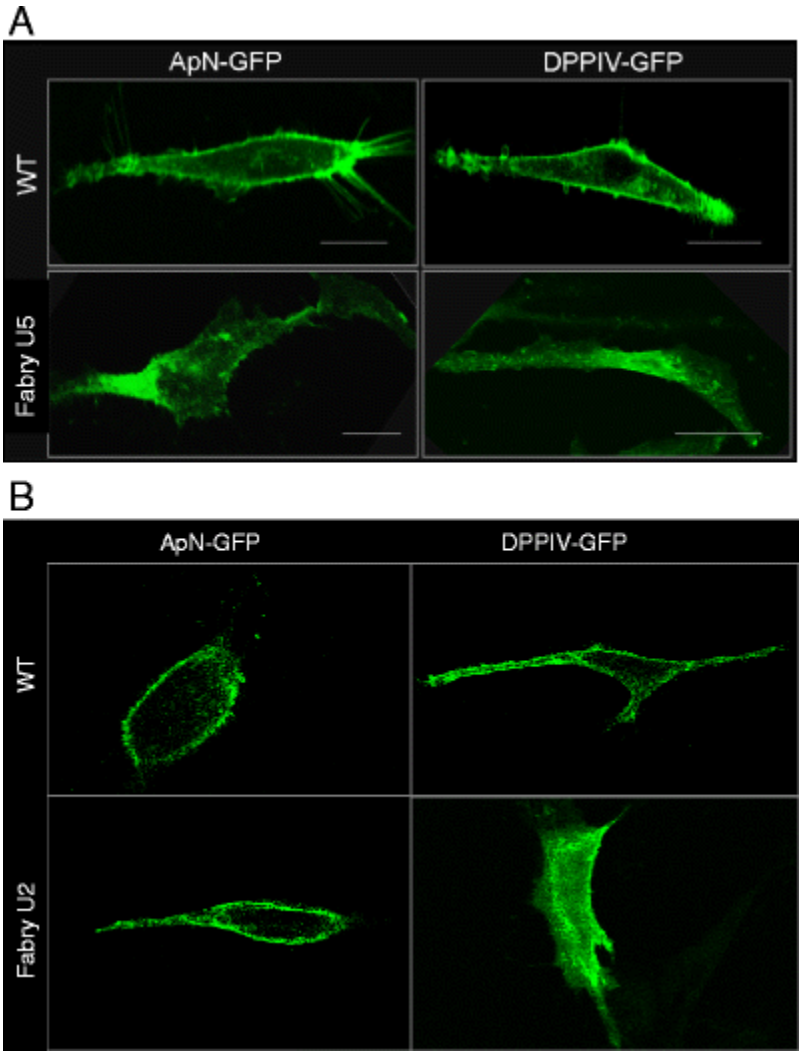

Figure 3 Subcellular localization of fusion forms of dipeptidyl peptidase IV (DPPIV) and aminopeptidase N (ApN) [DPPIV green fluorescence protein (GFP) and ApN-GFP] in transiently transfected wild-type (WT) and Fabry fibroblasts (U2 and U5). GFP-tagged forms of DPPIV and ApN were expressed transiently in U5 and U2 and WT fibroblasts. Note the cell-surface expression of DPPIV-GFP and ApN-GFP in WT fibroblasts (*upper panels* in A and B), whereas DPPIV-GFP (*lower right panels* in A and B), but not ApN-GFP, accumulates intracellularly in Fabry fibroblasts. *Scale bars* 20 µM

It has been previously shown that many lipids processed in lysosomes reside close to lipid-raft-related components, such as cholesterol and sphingomyelin, and some of them are processed in lysosomes (5,10). Therefore, the malfunctioning of α-galactosidase in Fabry disease strongly suggests that the membrane lipid composition is substantially altered, particularly in its contents of glycolipids. In fact, our data show that this is indeed the case. Furthermore, major consequences of these alterations are marked effects on the trafficking and sorting of a class of proteins, the DRM-associated proteins represented here by DPPIV. Other proteins that are transported to the cell surface via DRM-independent mechanism, such as ApN, remain unaffected by sphingolipid-related membrane alterations. The hampered trafficking of a population of proteins to the cell surface may lead to altered signalling at the cell surface triggered by DRM-associated proteins, with implications on gene regulation and subsequent protein expression.

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Chapter 3

M. Anderson-Fabry: Abnormalities of lipid rafts can be reversed by enzyme replacement or substrate reduction

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(Submitted)

Katia Maalouf's contributions to this article are the following:

- Design of individual experiments
- Performing experimental part of the study
- Analysis and interpretation of the results
- Writing manuscript

Abstract

Background: It is still not entirely clear how □-galactosidase deficiency translates into clinical symptoms of Fabry disease. Disturbed glycosphingolipid metabolism may lead to abnormal lipid composition of cell membranes resulting in alteration of membrane microdomains known as detergent-resistant membranes (DRMs) or lipid rafts.

Results: Alterations of lipid rafts were characterized in cultured fibroblasts derived from four male patients with Fabry disease and healthy controls. Here, the cells were solubilised with Triton X-100 and the distribution of flotillin 2 (a lipid rafts protein marker) in the floating lipid rafts fractions relative to soluble fractions in sucrose density gradients of the cellular lysates was assessed. In Fabry fibroblasts, flotillin 2 exhibited a significant shift to the soluble fractions of the sucrose gradient as compared to wild type cells compatible with a substantial change in the lipid composition of these cells. Incubation of the diseased cells with agalsidase alfa as enzyme replacement therapy elicited a significant shift of flottilin 2 from the soluble fraction to the floating lipid rafts fractions compatible with at least partial reversibility of lipid raft abnormalities in Fabry disease. Comparable results were obtained when diseased cells were treated with Miglustat (N-butyl-deoxynojirimycin (NB-DNJ)), an inhibitor of glucosylceramide synthase, strongly suggesting that alterations of lipid rafts are related to lysosomal storage phenomena.

Conclusions: Abnormalities in the distribution of flottilin 2, a lipid raft-associated protein, were observed in fibroblasts from males with Fabry disease. Since lipid rafts play a key role in various signalling pathways of cells, our observations may indicate that structural alterations in cellular

membranes contribute to the pathophysiology of M. Fabry. Miglustat or enzyme replacement therapy with agalsidase alfa is potentially capable of reversing these abnormalities.

Keywords: Morbus Anderson-Fabry, N-butyl-deoxynojirimycin, agalsidase alfa, enzyme replacement therapy, fibroblasts

Abbreviations: AGAL, alpha-galactosidase A; ApN, aminopeptidase N; DPPIV dipetidylpeptidase IV; DRM, detergent-resistant membranes; NB-DNJ, N-butyl-deoxynojirimycin; ERT Enzyme replacement therapy; FD Fabry disease; SRT: substrate reduction therapy; LSDs, Lysosomal storage diseases; Gb3, globotriaosylceramide

Background

Fabry disease (FD, MIM ID # 301500) – also known as Anderson-disease in the anglo-american literature - is a multisystemic disease caused by deficiency of the lysosomal enzyme alpha-galactosidase A (AGAL) (1). It is inherited as an X-linked trait, therefore the phenotype is generally more severe in male hemizygotes as compared to female heterozygotes (2). Characteristic clinical features are painful episodic crises, acroparesthesias, angiokeratoma, cardiac dysfunction, kidney disease, cerebrovascular events, cornea verticillata and gastrointestinal symptoms (3).

As a result of AGAL-deficiency, biodegradation of glycosphingolipids is disturbed, which leads to the intralysosomal accumulation of terminal galactosyl-containing neutral lipids (4). Lipid storage is not limited to lysosomes, even in plasma elevated concentrations of glycosphingolipids were found (5). Storage phenomena can be observed already in the placenta

of affected patients (6), however clinical symptoms set in with a delay of several years in most patients (2, 3). Thus, clinical symptoms are not directly related to storage of glycolipids, other functional disturbances must occur.

In previous studies, we have shown secondary biochemical alterations in Fabry cells: 1) trafficking of proteins associated with detergent resistant membranes (DRM) to the cell surface is hampered, which may lead to altered signalling at the cell surface (7). 2) secondary dysfunction of mitochondrial energy metabolism does also occur in fibroblasts from FD-patients (8).

Lipid rafts are ordered liquid domains rich in sphingolipids and cholesterol segregated from less-ordered liquid domains composed of mainly unsaturated phospholipids. Thus, lipid rafts depend on lipid membrane composition. Nowadays, there is increasing evidence for a regulatory role of these lipid rafts particularly in signalling and trafficking pathways (9,10). It is still not clear if therapeutic interventions in case of FD could modulate secondary biochemical alterations e.g. changes in lipid raft organization.

Enzyme replacement therapy (ERT) is a therapeutic option which leads to rapid (i.e. less than 2 days) elimination of storage material in patients with FD (11). In the literature, complete loss of storage material from FD-fibroblasts after 9 days of incubation with crude AGAL was reported (12). Incubation with commercially available agalsidase alfa resulted in even faster disappearance of storage material (13). This is in contrast to the clinical response which takes months to years. Two enzyme preparations are available on the European market (agalsidase

alfa and agalsidase beta), which are both reasonably safe and efficient in most patients (14, 11), however in others lack of improvement or even progression of disease does occur (5, 15-17).

An alternative to ERT is substrate reduction therapy (SRT) which involves the inhibition of glycosphingolipid synthesis and thus reducing substrate influx to the lysosome and restoring the balance between the rate of biosynthesis and the rate of catabolism (18). SRT uses an orally available, small molecule drug that inhibits the first committed step in glycosphingolipid biosynthesis (19). N-butyldeoxynojirimycin (NB-DNJ) (OGT 918, miglustat, Zavesca® manufactured by Actelion) inhibits glucosylceramide-synthetase. This substance is commercially available for the treatment of two other lysosomal storage diseases M. Gaucher, M. Niemann-Pick type C (NPC). This small molecule is able to cross the blood–brain barrier and thus can influence intracerebral manifestations (4).

In the present study, we characterize alterations of lipid rafts in fibroblasts derived from M. Fabry patients compared to wild type cells. The cultured fibroblasts were treated with agalsidase alfa (thereby clearing the cells from storage material) in an attempt to reverse secondary biochemical abnormalities. By using SRT with NB DNJ we address the question whether reversal of biochemical abnormalities is related to lysoamal storage of glycolipids.

Methods

Patients:

Fibroblasts from 4 adult male patients with genetically proven FD (for mutations see Table 1) were used for this study. Cell lines were primarily established for diagnostic purposes. Control

cells were taken from our tissue bank. The study was approved by our local ethics review board. All patients gave informed consent for this study.

Table 1 Mutations in fibroblasts from 4 male M. Fabry patients

	Mutation	**Amino-acid change**
Patient 1	**c.644 A>G**	**N215S**
Patient 2	**c.320 A>T**	**D107I**
Patient 3	**c.1083 G>A**	**G361X**
Patient 4	**c.658 C>T**	**R219X**
	c.320 A>T	**D107I**

Cell culture

Cultured skin fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (PAA, Coelbe, Germany) supplemented with 10% fetal bovine serum (Gold, Sigma), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C, 95% humidity, and 5% CO_2. Cells were from early passage numbers (max. number: 5).

Reagents

Tissue culture material was purchased from Biochrom (Berlin, Germany), FALCON 3001 tissue culture dishes were from Becton-Dickinson (Heidelberg, Germany). All chemicals used came from Sigma-Aldrich (Taufkirchen, Germany), enzymes were obtained from Roche (Mannheim, Germany). Flotillin2 and RhoA antibodies were purchased from Santa Cruz. Agalsidase alfa

(Replagal) was generously provided by Shire, and N-butyl-deoxynojirimycin (NB-DNJ)/Miglustat was kindly provided by Actelion Pharmaceuticals, Germany.

Treatment of cells

For the analysis of lipid-rafts, fibroblasts were plated on Petri dishes (diameter 60 x15 mm) and grown to confluence. They were then incubated with 10 and 20 µg/ml agalsidase alfa or with 50 and 100 µM NB-DNJ for three days with daily changes of incubation medium. These concentrations were chosen based on the studies in which clearance of storage material was demonstrated (13, 20, 12).

Lipid rafts

After 72 hours of incubation, lipid rafts were prepared from cells grown to confluence in 60x15 mm dishes. All solutions and materials were cooled down to 0-4 °C and handling was done at 0-4 °C. After two washes with PBS, cells were scraped-off into 500 µl raft buffer (1% Triton X-100 in PBS) containing protease inhibitors (Pepstatin, leupeptin, aprotinin, and trypsin inhibitor were obtained from Sigma, and soybean trypsin inhibitor from Roche Diagnostics). Homogenization was carried out by passing the cell lysate 20 times through a 21G needle. The homogenates were then kept shaking for three hours at 4ºC. Afterwards, the lysates were adjusted to 40% w/v sucrose by addition of 1 volume of 80% w/v sucrose prepared in PBS. A discontinuous gradient was formed: (1ml 80% w/v sucrose, 1ml 40% w/v lysate, 7 ml 30% w/v sucrose, and 1ml 5% w/v sucrose). Gradients were centrifuged at 4 °C and 100,000 x g for 18 h using a Beckman-centrifuge equipped with a SW40 rotor. Nine fractions were collected at 4 °C from bottom to top.

Statistical analysis

Western blot bands were quantified using ImageJ software. Data were analysed by using Excel 2003 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). Data from enzyme treated and untreated fibroblasts were compared using Student's t-test for paired data. A p-value < 0.05 indicated a significant difference.

Results and discussion

Glycolipids which are components of lipid rafts -along with cholesterol and sphingomyelin- accumulate in FD due to malfunctioning of the lysosomal enzyme alpha-galactosidase. This may imply that membrane homeostasis in Fabry cells is disrupted leading to impairment of trafficking and sorting of lipid raft-associated proteins and disturbance of the overall protein–lipid interaction at the cell membrane. As a result, cell surface signaling, gene regulation, and subsequent protein expression may be altered (21).

In this study, alterations of the lipid raft marker flotillin 2 were determined using sucrose-based density gradients for Triton X-100 DRMs, followed by immunoblotting with an antibody against flotillin 2. From this sucrose gradient nine fractions were collected out of which the first four fractions were considered to be the floating fractions containing the lipid rafts. This was based on the pattern seen in wild type cells where flotillin 2 floated in the first four fractions of the gradient (Fig1 A, wild type). The trafficking of lipid raft associated protein flotillin 2 appeared to be altered in Fabry cells based on the apparent reduction of this protein in the floating fractions (fractions 1 to 4) of the gradient. At the same time, flotillin 2 accumulated in the soluble fractions (fractions 8 and 9) of the gradient (Fig1, A). The mean value of flottilin 2 in the cellular lysates of the four patients revealed a highly significant two fold decrease in the

percentage of flotillin 2 in the floating fractions versus the soluble fractions (Fig1, B). This finding supports the notion that lipid rafts are distorted and subsequently implicated in the pathophysiology of FD.

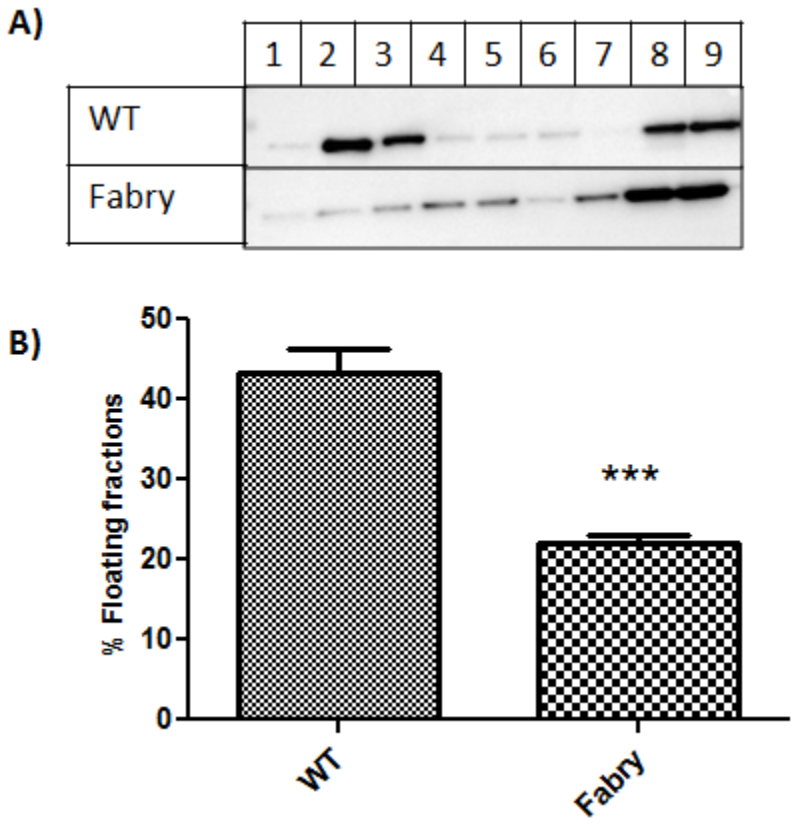

Figure 1 Alterations of lipid rafts of fibroblasts derived from Fabry patients. Fibroblasts derived from Fabry patients or healthy individuals were lysed with 1% (w/v) Triton X-100 and run on density-based sucrose gradients. Nine fractions were collected and analyzed for distribution of flotillin 2 by immunoblotting. A) Representative immunoblots from patient 1showing distribution of flotillin 2 in above-mentioned gradient fractions. B) The mean value ± SEM of flotillin 2-distribution in the floating fractions was calculated for all four patient fibroblasts and four control fibroblasts. The percent of flotillin 2 in the floating fractions decreased from 44% in WT to 22% in M. Fabry fibroblasts (***$p<0.001$).

As a control experiment, sucrose density gradient fractions were immunoblotted with RhoA which is a non-lipid rafts-associated protein. RhoA appeared only in the soluble fractions (fractions 8 and 9) of the gradient in wild type and in all patient fibroblasts (Fig2), indicating that the trafficking of non-lipid rafts-associated proteins is not affected in Fabry fibroblasts.

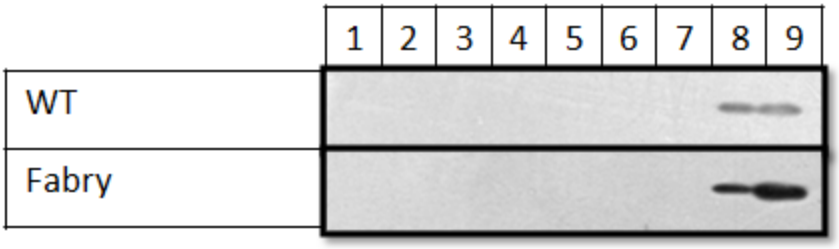

Figure 2 Distribution of the non-lipid rafts-marker RhoA in Triton x-100-treated fibroblasts derived from Fabry patients and healthy controls. Sucrose density gradient followed by immunobloting with RhoA antibody revealed that the non-raft-associated marker RhoA appears to be present in the soluble fraction (fractions 8 and 9) of the gradient in all wild type and all patient cell lines. Representative immunoblots from patient 1 is shown.

Interestingly, when the Fabry cells were incubated with agalsidase alfa as an enzyme replacement, i.e. ERT for mutated α-galactosidase, a partial restoration of lipid rafts was observed revealed by the shift of flotillin 2 back to the floating fractions of the gradients (Fig3, A). ERT is currently the therapy of choice for males and females with Fabry disease resulting in beneficial effects on symptoms like neuropathic pain, nephropathy, cardiomyopathy, and gastrointestinal symptoms as well as quality of life (22). In addition, stabilization of hearing and improved thermal sensations has been shown by Hajioff et al in 2003 (23). In line with our findings in Fabry fibroblasts Machann and coworkers demonstrated compromised cardiac energy metabolism in Fabry patients which improved under ERT (24). This can be taken as a proof of principle that at least certain biochemical findings in fibroblasts in vitro can be

extrapolated to the clinical setting. Treatment of Fabry cells with α-galactosidase in the present study only partially restored lipid rafts; the maximum shift of flotillin 2 back to the lipid rafts floating fractions was around 30% on average as compared to 44% of normal flottilin 2 levels in the floating fractions of wild type cells (Fig3, B). This partial recovery may be related to incomplete clinical response of some patients to ERT.

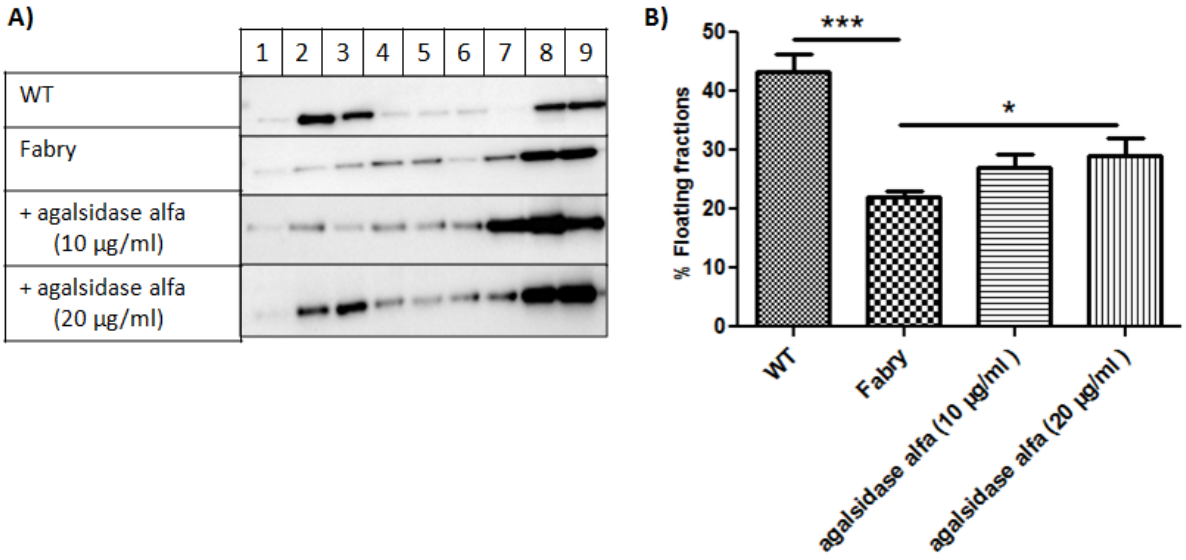

Figure 3 Effect of enzyme replacement therapy on alterations of lipid rafts of fibroblasts derived from Fabry patients. Fibroblasts derived from Fabry patients were incubated with 10 and 20 µg/ml agalsidase alfa for three days, after which Triton X-100 DRMs were extracted and run on sucrose density gradients followed by immunoblotting and analysis of flotillin2 distribution as described above. A) Representative immunoblots from patient 1showing distribution of flotillin 2 in above-mentioned gradient fractions. B) The mean value ± SEM of flotillin 2-distribution in the floating fractions was calculated. Treatment of fibroblasts from the four Fabry fibroblasts with agalsidase alfa resulted in a shift of flotillin 2 from soluble fractions back to the floating fractions of the sucrose density gradient up to 29 % with 10 µg/ml and 31% with 20 µg/ml of agalsidase alfa (*$p<0.05$).

In principle, the effect of ERT on lipid rafts may be independent from reduction of storage material. SRT via the glucosylceramide synthase inhibitor NB-DNJ (Miglustat) is another option

to reduce storage material in the cell. Treatment of Fabry cells with 100 µM NB-DNJ led to a significant restoration of flotillin 2 into the DRM fractions up to an average of 35% (Figure 4). This compound was effective even at the lower dose used (50 µM), in line with previous work by Platt el 1994 (25) where 50 µM of NB-DNJ was enough to reduce glucosylceramide (GlcCer) to levels similar to wild type cells. Thus, we showed that the effect of ERT on lipid rafts is likely to be related to the reduction of storage phenomena. Miglustat also exhibits a chaperone activity in addition to its SRT function and is therefore capable of modulating the activity of α-galactosidase by supporting the proper folding and thus trafficking of certain misfolded enzymes to their functional site in the lysosomes (26). Thus, the chaperone function of Miglustat may play an additional role in our study by partially rescuing α-galactosidase activity in Fabry disease. Previously, an almost 9-fold increase of α-galactosidase activity was demonstrated in Fabry fibroblasts when α-galactosidase (rhGLA, Fabrazyme) was coincubated with 1-deoxygalactonojirimycin (DGJ), a galactose analogue with chaperone activity. These results demonstrated the enhancing effect of chaperones on recombinant enzymes used for ERT (27). In a recent in silico study, it has been shown that not all mutations respond to chaperone therapy (28). Even in those cells responding to the chaperone activity of Miglustat the restored enzyme activity was substantially lower than its wild type counterpart. Although the mutations present in our patients were not among the mutations tested in that study, we assume that the Miglustat effects observed here were mainly due to substrate reduction.

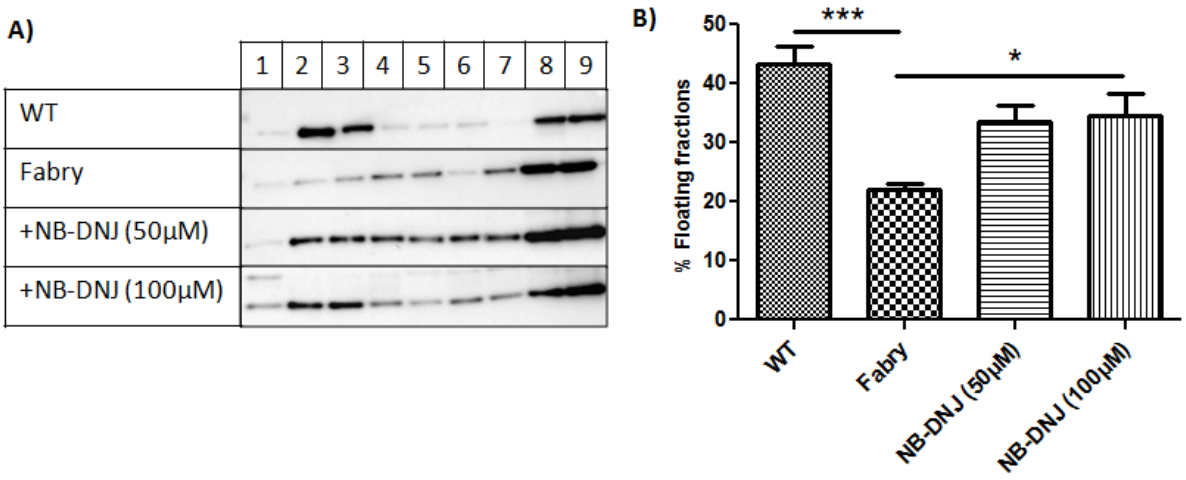

Figure 4 Effect of NB-DNJ on alterations of lipid rafts of fibroblasts derived from M. Fabry patients. Fibroblasts derived from Fabry patients were incubated with 50 and 100 µM NB-DNJ for three days, after which Triton X-100 DRMs were extracted and run on sucrose density gradients followed by immunoblotting and analysis of flotillin2 distribution as described above. A) Representative immunoblots from patient 1 showing distribution of flotillin 2 in above-mentioned gradient fractions. B) The mean value ± SEM of flotillin 2-distribution in the floating fractions was calculated. Treatment of fibroblasts from the four Fabry fibroblast cell lines with NB-DNJ resulted in a shift of flotillin 2 from soluble fractions back to the floating fractions of the sucrose density gradient up to 33 % with 50 µM and 35% with 100 µM of NB-DNJ (*$p<0.05$).

Conclusions

Lipid rafts are enriched in glycolipids and cholesterol. They are critical components of cellular membranes and participate in several essential intracellular signalling mechanisms that regulate cell growth, survival and development. It is plausible to assume that altered concentrations of either of these components, cholesterol or glycolipids, distort the integrity of lipid rafts with an ultimate impairment of function. In this study, we could demonstrate that fibroblasts derived from male patients with Fabry disease exhibit abnormalities in lipid rafts. This is supported by two observations: Firstly, the levels of the rafts protein marker, flottilin 2,

in the floating fractions of cellular lysates of Fabry cells are substantially reduced as compared to their control counterparts compatible with an overall reduction of these membrane microdomains. Secondly, an increase in the levels of flottilin 2 in the floating fractions upon incubation with either Miglustat or ERT is observed pointing to at least partial restoration of the membrane microdomain composition. We have previously shown that lipid rafts-associated proteins are blocked intracellularly in Fabry fibroblasts (7). Thus, abnormalities in lipid rafts in the plasma membrane affect membrane and protein trafficking, interfere or impair the function of proteins due to mistargeting and contribute thus to the pathophysiology of Fabry disease.

Lipid rafts play a role in the pathogenesis of several genetic diseases. For example, hereditary nephritic syndrome is elicited by the disruption of nephrin targeting to lipid rafts due to a mutation in the NPHS2 gene (29). Likewise, the failure of the intestinal enzyme sucrase-isomaltase to associate with lipid rafts blocks the trafficking of this protein to the apical membrane in the enterocytes eliciting thus carbohydrate malabsorption in some cases of congenital sucrase-isomaltase deficiency (30). Finally, the CFTR in patients with cystic fibrosis due to the DeltaF508 mutation is not associated with lipid rafts and thus not further trafficked in the cell (31).

While structural alterations due to mutations in proteins hamper their association with otherwise normal lipid rafts in these diseases, our study provides a new mechanism in which the lipid rafts are distorted and their correction with either ERT or Miglustat leads to partial restoration of trafficking. The (only) partial recovery explains the persistence of clinical

symptoms under ERT in some patients. As Miglustat which leads to substrate reduction had a similar effect as ERT, we assume that the observed effects on lipid rafts are mainly associated with a reduction of cellular storage material.

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Authors' contributions

KM performed experiments, interpreted results, drafted the manuscript. MvK-B provided helpful scientific feedback, drafted and revised the manuscript. HYN and AMD designed the experiments, interpreted results and revised the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Chapter 4

M. Niemann Pick C1: Characterization of lipid raft abnormalities and the reversing effects by N-butyl-deoxinojirimycin in a case of M. Niemann Pick C1

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In preparation for submission

Katia Maalouf's contributions to this article are the following:

- Design of individual experiments
- Performing experimental part of the study
- Analysis and interpretation of the results
- Writing manuscript

Summary

One potential mechanism leading to clinical symptoms in Morbus Niemann –Pick type C1 (NPC1) may be the altered composition of cellular membrane lipids with subsequent impairment of lipid raft function and protein trafficking. Cholesterol-and sphingolipid- enriched membrane microdomains (lipid rafts) were found to be altered in fibroblasts derived from a NPC1 patient as assessed by the altered distribution of flotillin 2 (a protein marker of lipid rafts) in Triton X-100 lysates. We also found a substantial increase in dipeptidyl peptidase IV (DPPIV) (a protein known to be associated with lipid rafts) levels in the patient's fibroblasts. Endocytosis of DPPIV appeared to be slower in NPC1 cells compared to healthy cells. N-butyl-deoxynojirimycin (NB-DNJ), an N-alkylated imino-sugar which inhibits the ceramide-specific glucosyltransferase has been advocated in the treatment of NPC. We incubated NPC1 cells with NB-DNJ and examined its effect on the above mentioned phenotypes. NB-DNJ was able to restore flotillin 2 distribution, and by enhancing endocytosis levels, it was capable of decreasing DPPIV levels on the cell surface of NPC1 cells. Our study demonstrates that impaired lipid rafts play a major role in pathogenesis of NPC1 disease and that NB-DNJ as a potential therapeutic agent for NPC patients reverses membrane lipid abnormalities in NPC1 fibroblasts.

Key words: Neimann Pick type C, Lipid rafts, pathophysiology, endocytosis.

Abbreviations: NPC1, Morbus Niemann–Pick type C1; LSD, lysosomal storage disease; DPPIV, dipeptidyl peptidase IV; NB-DNJ, N-butyl-deoxynojirimycin; CNS, central nervous system; DRM, detergent-resistant membrane; DMEM, Dulbecco's modified Eagle's medium; HRP, Horseradish peroxidase; EEA-1, early endosomal antigen-1;

PBST, PBS-0.05% Tween 20; RT, room temperature; PBS, phosphate-buffered saline; Lo, liquid-ordered; Ld, liquid-disordered; ApN, aminopeptidase N; glycosphingolipids, GSL

Introduction

The clinical picture of Morbus (M.) Niemann-Pick was first described in 1920s by Albert Niemann and Ludwig Pick. Niemann-Pick C1 (NPC1) disease is characterized by a defect in trafficking of endocytosed cholesterol with sequestration of unesterified cholesterol in lysosomes and late endosomes (1). Other lipids also accumulate with variation between different tissues: cholesterol and sphingomyelin storage is most abundant in peripheral tissue, whereas glycosphingolipid (GSL) buildup is most significant in the central nervous system (CNS) (2).

NPC disease affects the viscera (enlarged liver, spleen, and sometimes lung) and central nervous system with most common symptoms (over 70% of cases) being clumsiness, learning difficulties ataxia, dysphagia, dysarthria and vertical gaze palsy (a disease defining symptom) (3). The clinical spectrum of NPC ranges from a neonatal rapidly fatal disorder to an adult-onset neurodegenerative disease. Its incidence is approximately 1/120 000 live births, however due to lack of clinical symptoms specificity, NPC is often misdiagnosed or remains undetected (1). Primary diagnosis methods involve a biochemical test which involves staining cultured fibrobalsts from skin biopsies with filipin (a fluorescent sterol binding compound) that detects lipid accumulation in the lysosomes. Genetic testing is usually done to confirm primary diagnosis (4).

NPC is caused by an autosomal recessive mutation in one or both of the genes: NPC1 which is the main focus of our study and NPC2. The precise function of these two proteins remains unclear, yet there is increasing evidence that both together coordinate to transport cholesterol and other compounds out of the late endosome/ lysosome system (1). NPC1 gene which accounts for the majority of identified mutations (95%) is located on chromosome 18q11–12, it has a size of approximately 4.9-kb messenger RNA which translates into a 1278-amino-acid protein. NPC1 protein is a multi-transmembrane protein localized in the late endosomes/lysosomes system. NPC1 function was originally thought to be mainly associated with cholesterol trafficking. Recently however, more evidence is pointing out that NPC1 may be involved in a retrograde transport of various lipids and proteins from late endosomes to the TGN (5).

A promising therapeutic option of NPC is substrate reduction by compounds like N-butyldeoxynojirimycin (NB-DNJ) which inhibits glycosphingolipids synthesis leading to amelioration of storage processes. This glucose analogue is small and can cross the blood brain barrier and thus can influence intracerebral manifestations. NB-DNJ has been approved by the European Union and elsewhere for the treatment of NPC and M. Gaucher patients. Symptomatic management of patients is also crucial.

The precise mechanism for pathogenesis of NPC disease still remains unclear. Since NPC is a cholesterol and glycolipid storage disease, it can be hypothesized that NPC is associated with alteration of membrane subdomains known as lipid rafts. Lipid rafts are ordered liquid domains rich in sphingolipids and cholesterol segregate from less-ordered liquid domains composed of

mainly unsaturated phospholipids. They can be extracted using nonionic detergents and thus are also called detergent-resistant membranes (DRMs). There is increasing evidence for a regulatory role of these lipid rafts particularly in signaling and trafficking pathways (6, 7, 8).

In this study we characterized lipid rafts abnormalities in fibroblasts derived from an adult female patient with genetically proven NPC1 disease (9). Furthermore we investigated the effect of NB-DNJ on its ability to reverse detected abnormalities.

Materials and Methods

Cell culture

Cultured skin fibroblasts from a female patient with genetically proven NPC1 disease and four healthy age-matched males were grown in Dulbecco's modified Eagle's medium (DMEM) (PAA, Coelbe, Germany) supplemented with 20% fetal bovine serum (Gold, Sigma), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C, 95% humidity, and 5% CO_2. Cells were from early passage numbers (max. number: 15).

Reagents

Tissue culture material was purchased from Biochrom (Berlin, Germany), FALCON 3001 tissue culture dishes were from Becton-Dickinson (Heidelberg, Germany). All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). Pierce® Cell Surface Protein Isolation Kit was purchased from Thermo Scientific (Munich, Gemany). NB-DNJ (OGT 918, Miglustat, Zavesca®) was kindly provided by Actelion Pharmaceuticals, Germany. Streptavidin-Horseradish peroxidase (HRP) conjugate was from GE Healthcare (Weissling, Germany).

Antibodies

Flotillin2 and RhoA antibodies were purchased from Santa Cruz, Heidelbegr, Germany. Early endosomal antigen-1 (EEA-1) derived from Dianova (Hamburg, Germany). The monoclonal Dipeptidyl peptidase IV (DPPIV) antibody for Western blot was a generous gift from Hans-Peter Hauri, Biozentrum, Basel and Erwin Sterchi, University of Bern. A FITC labeled CD26 antibody for FACS analysis was purchased from BD Biosciences (Heidelberg, Germany). The secondary antibody ImmunoPure Goat Anti-Mouse IgG, (H+L), peroxidase conjugated was purchased from Thermo Scientific (Munich, Germany). Goat Anti-mouse AlexaFlour488 IgG (H+L) and goat anti-rabbit AlexaFlour568 IgG (H+L) were purchased from Invitrogen (Darmstadt, Germany).

Treatment of cells

For the isolation of cell surface proteins, fibroblasts were plated in Petri dishes (diameter 100 x 20 mm) and after 24 hours of growth they were incubated with 50, 100 and 200 μM NB-DNJ for three days with daily changes of incubation medium. These concentrations were chosen based on the studies in which clearance of storage material was demonstrated (10).

For conducting FACS analysis, for lipid-rafts isolation and for endocytosis assay, fibroblasts were plated in Petri dishes (diameter 60 x15 mm) and after 24 hours they were incubated with above mentioned concentrations of NB-DNJ for three days with daily changes of incubation medium.

Protein isolation, quantification and Western blot analysis

Total protein amounts were extracted from NPC1 and WT fibroblasts using lysis buffer (25 mM Tris-HCl, 50 mM NaCl, 0.5% DOC and 0.5% Triton X-100) supplemented with protease inhibitors (1 mM PMSF, 1 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml antipain and 50

µg/ml trypsin-chymotrypsin inhibitor) Western blot analyses were performed with equal amounts of total protein. Protein concentrations in the lysates were determined by using the Bradford protein assay kit (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. Proteins were then separated by SDS-PAGE on 6% polyacrylamide gels (11) and later transferred to PVDF membranes which were blocked overnight in 5 % milk in PBS, 0.05% Tween 20 (PBST) at 4 °C. Membranes were incubated with primary antibodies anti-DPPIV 1:1000 for 1 h in 2 % milk in PBST at room temperature (RT) and washed three times for 10 min in PBST. Secondary antibody anti-mouse-HRP (Thermo Scientific) was then incubated for 1 h in 2 % milk in PBST at RT and washed three times for 10 min in PBST. Proteins were measured using enhanced chemiluminescence with SuperSignal West Femto Chemiluminescent Substrate (Thermo scientific) and the ChemiDoc system (Bio-Rad, Munich, Germany) with QuantityOne software (Bio-Rad). Quantification of protein levels was performed with "ImageJ 1.41i" and "Microsoft Office Excel 2003".

Cell surface protein isolation

After three days of NB-DNJ incubation and after reaching 90-95% confluency, cell surface proteins were isolated according to Pierce® Cell Surface Protein Isolation Kit according to the manufacturer's instruction manual with some modifications. Briefly, cells were washed twice with ice cold phosphate-buffered saline (PBS) and incubated with 10ml 0.25 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Thermo Scientific, Bonn, Germany) in PBS for 30 min gently shaking on ice. The reaction was stopped by adding 500 µl Tris (50 mM, pH 8). Cells were scraped and collected in 15 ml Falcon tubes and centrifuged at 500 x g for 5 min. The cell pellets were washed with 5 ml Tris-buffered saline (TBS; 25 mM Tris, 0.15 M sodium chloride, pH 7.2) and later centrifuged

at 500 g for 5 min. After discarding the supernatants, cell pellets were resuspended in lysis buffer (25 mM Tris-HCl, 50 mM NaCl, 0.5% DOC and 0.5% Triton X-100) supplemented with protease inhibitors (same as before). Cell lysates were disrupted by sonicating on ice using five 1-second bursts, and incubated 30 minutes on ice, with vortexing every 5 minutes for 5 seconds. Samples were centrifuged at 10.000 x g for 2 min and the clarified supernatants were incubated with 100 µl Neutravidin agarose beads (Thermo Scientific, Bonn, Germany) for 1 h shaking at RT. At the end of incubation time, beads were washed two times with PBS, 0.5% Triton X-100 and 0.05 % NaDOC and twice with 500 mM NaCl, 125 mM Tris, 10 mM EDTA and 0.5% Triton X-100 (pH 8). Cell surface proteins were eluted by incubating the Neutravidin beads with sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol) with 50 mM DTT final concentration 1 h shaking at RT followed by a centrifugation for 2 min at 1.000 x g. Protein determination and Western blots were performed as described above.

Endocytosis immunofluorescence assay

Fibroblasts were grown on coverslips to a confluence of maximum 60%. On the day of experiment, cells were kept on ice to stop endocytosis, they were then washed and incubated with primary DPPIV antibody in medium (1:500) and kept shaking on ice for one hour. After that, one group of cells were kept on ice and the other group were given new pre-warmed medium and transferred to a 37 °C incubator for 30 min to again induce endocytosis. Subsequently, the cells were rinsed twice with PBS and then fixed with 4% paraformaldehyde (PFA; Fluka BioChemika, Buchs, Switzerland) for 40 min and further processed as previously described (11). In brief, the cells were quenched for 30 min shaking at room temperature with 50mM ammonium chloride in PBS, followed by incubation with a blocking buffer (0.5% Saponin

(Sigma Aldrich Chemie, Taufkirchen)) in PBS and 1% BSA (Fluka BioChemika, Buchs, Switzerland) for 30min. As secondary antibody AlexaFlour488 was utilized at 1:1000 dilution. EEA-1 (1:200) was used for the detection of early endosomes, followed by secondary labeling with goat anti-rabbit AlexaFlour568 (1:1000). Finally, the cells were washed with PBS and mounted with mowiol 4–88 (Calbiochem, an Affiliate of Merck, Darmstadt, Germany).

Confocal fluorescence microscopy

Confocal images of fixed cells were acquired using a Leica TCS SP5 microscope with a 63x oil Planapochromat lens (Leica Microsystems, Wetzlar). Dual color AlexaFlour488 and AlexaFluor568 images were obtained by sequential scans with the 468 nm excitation line of an argon laser or the 543 nm excitation line of a He/Ne laser, respectively (12).

Cell surface biotinylation assay for endocytosis

A cell surface biotinylaion endocytosis assay was conducted as described by Liu et al. 1998 (13). Briefly, cells grown to confluence in culture dishes (diameter 60 x15 mm), during which NPC1 cells were incubated with 100 µM NB-DNJ for three days. On the day of experiment, cells were washed with pre-cooled PBS containing 1 mM magnesium chloride and 1 mM calcium chloride. NHS-SS-Biotin (Thermo Scientific) dissolved in PBS (0.5mg/ml) was added to cells and kept for 20 min at 4°C with shaking. Cells were then washed with DMEM-BSA (1%) and two control cell plates were kept on ice whereas the others were transferred to 37°C incubator to induce endocytosis event again: One control dish reduced (0%) and one not reduced (100%). Incubation at 37°C was stopped after various time points by returning the cells back on ice after which the cells were washed with 10% FBS in PBS. To remove the remaining surface biotin, cells were incubated with the reducing solution (310mg glutathione-free acid in 17ml ddH2O

with 1ml of 1.5M NaCl, 0.12ml of 50%NaOH, and 2ml serum) twice for 20 min. After that cells were then washed with PBS and incubated for 15 min with iodoacetamide (5mg/ml) in 1% BSA in PBS for 15 min to quench any free sulfhydryl groups. Cells were then lysed with standard lysis buffer supplemented with protease inhibitors as mentioned above. DPPIV was immunoprecipitated in the presence of protein A-sepharose. The immuno-isolates were further processed by non-reducing SDS-PAGE followed by immunoblotting with streptavidin-HRP as described above.

Lipid rafts

Lipid rafts were prepared from 72 hour treated cells grown to confluence in 60x15 mm dishes. All solutions and materials were cooled down to 0-4 °C and handling was done at 0-4 °C. After two washes with PBS, cells were scraped into 500 µl raft buffer (1% Triton X-100 in PBS) containing protease inhibitors as described before. Homogenization was carried out by passing the cell lysate 20 times through a 21G needle. The homogenates were then kept shaking for three hours at 4ºC. After that the lysates were adjusted to 40% w/v sucrose by addition of 1 volume of 80% w/v sucrose prepared in PBS. A discontinuous gradient was formed: (1ml 80% w/v sucrose, 1ml 40% w/v lysate, 7 ml 30% w/v sucrose, and 1ml 5% w/v sucrose). Gradients were centrifuged at 4 °C with 33,000 rpm (100,000 x g) for 18 h using a Beckman-centrifuge with a SW40 rotor. Nine fractions were collected at 0-4 °C from bottom to top; fractions nine and eight typically contained the detergent-soluble cell pellet, and fractions one to four contained the DRMs/lipid rafts. Fractions were then separated on 12% for polyacrylamide gels for flotillin2/ RhoA immunoblots as described above.

Statistical analysis

Western blot bands were quantified using ImageJ software. Data were analysed by using Excel 2003 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). Statistical analysis was done using Student's t-test for paired data. A p-value < 0.05 indicated a significant difference.

Results and Discussion

This study was conducted on skin fibroblasts from a patient with genetically proven Niemman Pick C1 disease. This NPC1-patient is an adult female over which a case study was published by Grau et al. in 1997 (9). The *NPC1*-mutations were reported to be in Exon 18: 2744A>G (D874V) and in Exon 19: 2842G>A (D948Y).

Based on an imbalanced lipid composition in NPC1 disease, we hypothesized that the cell membrane composition and function in NPC1 cells would be altered, especially in microdomains called 'lipid rafts' that are known to be rich in these particular lipids. The tendency of cholesterol to pack tightly with saturated acyl chains of lipids allows the segregation of the lipid bilayer into a liquid-ordered (Lo) phase which is rich in cholesterol and glycosphingolipids and a liquid-disordered phase (Ld) which consists of phospholipids and other glycerophospholipid (14). The highly condensed state of these microdomains or 'lipid rafts' in the Lo state renders them resistant to solubilization by cold nonionic detergents such as Triton X-100. Their buoyant density, due to their higher lipid/protein ratio compared with bulk membrane, permits their flotation in and recovery from discontinuous sucrose gradients after isopycnic centrifugation (15). Here we investigated the distribution of flotillin 2 in Triton X-100 lysates of above-mentioned fibroblasts derived from a NPC1 patients compared to healthy

control cells. Flotillin 2 is well known as a marker for lipid rafts and the characterization of lipid rafts abnormalities (16).

The distribution of the 'lipid raft' associated protein flotillin2 was shown to be altered in the NPC1 patient cells by performing density based sucrose gradients for triton x-100 DRMs. A significant shift of this 'lipid raft' associated protein from the floating fractions 1 to 4 (representative of lipid rafts) in parallel with its accumulation in the soluble fraction 8 and 9 was detectable. This classification of 'lipid rafts' fractions was based on the repeated pattern obtained in four WT cells (fig1, A). In Figure 1, B the same procedure was repeated but this time the gradient fractions were immunoblotted with an antibody against RhoA (a non-raft-associated marker) as a control (17). RhoA appeared in both NPC1 and WT cells in the soluble fractions of the gradient (fig 1, B).

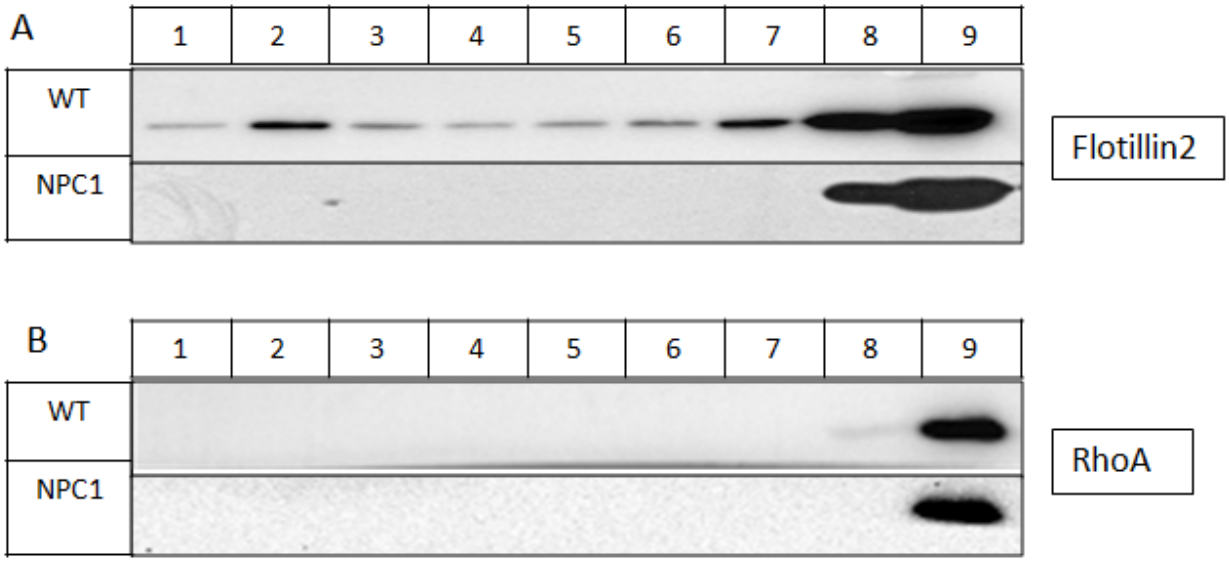

Figure1 Impaired distribution of 'lipid rafts' associated protein flotillin2 NPC1 cells. A) The distribution of the 'lipid raft' associated protein flotillin2 was shown to be altered by performing density based sucrose gradients for triton x-100, which revealed a significant decrease of this 'lipid raft' protein from

the floating fractions (1 to 4) and its accumulation in the soluble fractions (fractions 8 and 9). Representative immunoblots from one out of four WT cells and from the NPC-patient cells are shown. B) Sucrose density gradient followed by immunobloting with RhoA antibody revealed that this non-raft-associated marker RhoA appears to be present in the soluble fraction (fractions 8 and 9) of the gradient in wild type cells as well as patient cells. Representative immunoblots from one out of four WT cells and from NPC1 cells are shown. (* $p < 0.05$, $n \geq 3$ independent experiments).

DPPIV has been used several times by our group as a model for studying protein trafficking in health and disease and was previously shown in intestinal epithelial cells to be associated with cholesterol and sphingolipid rich microdomains (17, 18, 19). In another study on the implications of lipid rafts in Fabry disease, we were able to show that the trafficking of DPPIV to the cell surface was hampered in Fabry cells, whereas no effect was observed on the targeting of aminopeptidase N (ApN) that is not associated with this type of DRM (20). To examine the trafficking of DPPIV in NPC1 cells relative to normal fibroblasts, cell surface protein isolation was performed using a biotin-avidin system based assay. Here the proteins on the cell surface were labeled by incubating the cells with NHS-SS-biotin (a membrane impermeable, water soluble, amine-reactive reagent). The biotin tagged proteins were then precipitated with NeutrAvidin Agarose Resins which later were cleaved with a reducing along with the biotin tag leaving the cell surface proteins in the eluent. The (biotin-avidin) isolated cell surface proteins were then immunoblotted with an antibody against DPPIV (fig2, A) which revealed a significant 2-fold increase of this protein in the membranes of NPC1 cells relative to normal WT fibroblasts.

To verify these results using another method, FACS analysis was performed and the result revealed an apparent 2-fold accumulation of DPPIV on the surface of NPC1 compared to WT

cells (fig2, C). The total expression level of DPPIV was examined in both WT and NPC1 cells. Fig 2, B shows a Western blot for equal amounts of protein from total lysates with antibodies against DPPIV. In both normal and diseased cells, the overall level of expression of DPPIV appears unchanged.

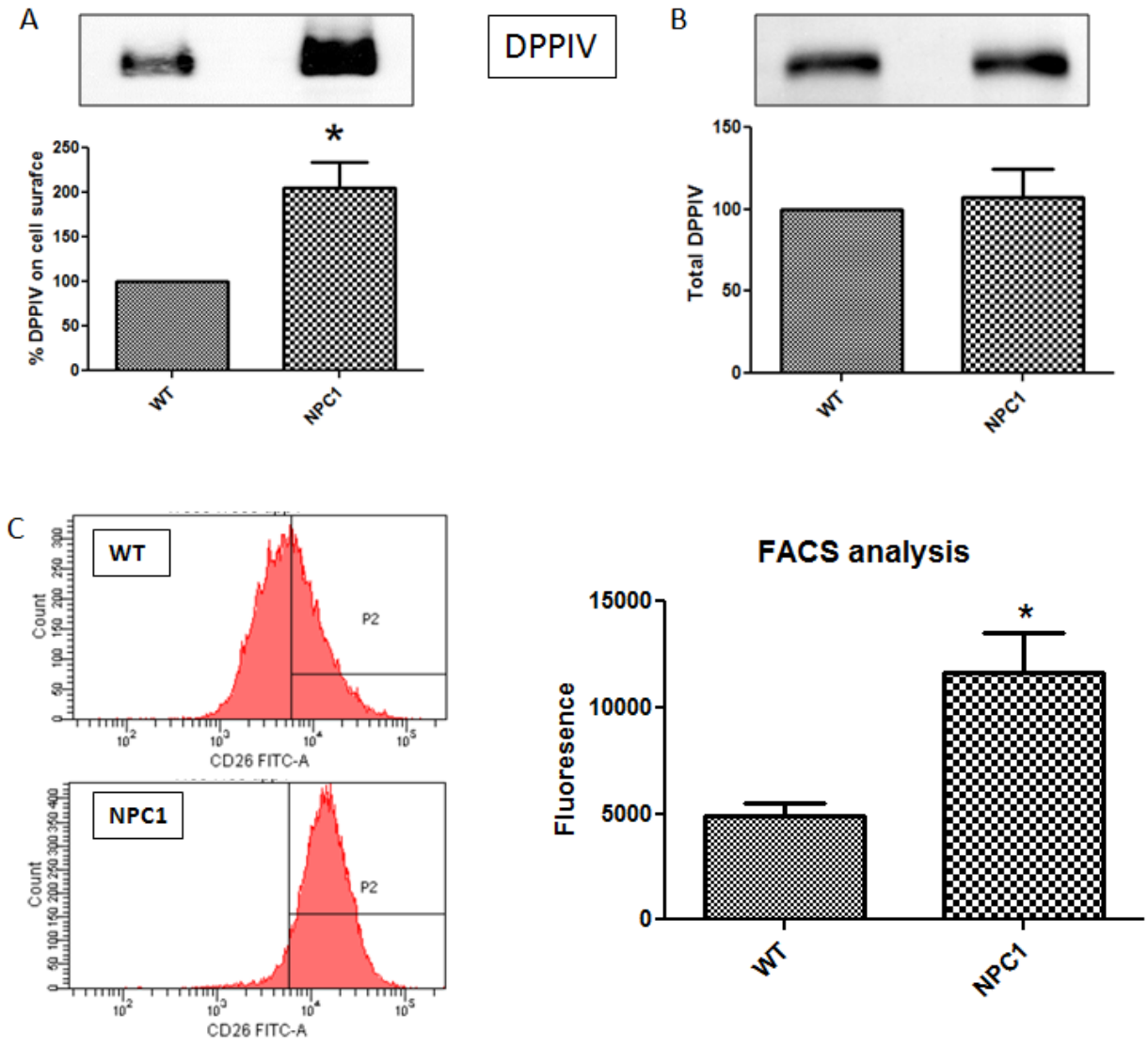

Figure 2 DPPIV accumulates on cell surface of NPC1 cells. Cell surface protein analysis was performed by: A) biotin-avidin system based assay where isolated cell surface proteins were then immunoblotted with a monoclonal DPPIV antibody, representative immunoblots from one out of four WT cells and from

patient1 are shown. and C) FACS analysis, where the increase in fluorescence signal emitted by NPC1 cells reflected an accumulation of DPPIV on the surface of NPC1 cells. A 2-fold increase of this protein was observed on the membranes of NPC1 cells relative to the mean value ± SEM of four normal WT fibroblasts. B) No significant difference in the total DPPIV expression levels appeared when immunoblotting with the same DPPIV antibody was done against total WT and NPC1 cell lysates. Representative immunoblots from one out of four WT cells and from patient1 are shown. (* $p < 0.05$, $n \geq 3$ independent experiments).

In an attempt to explain the mechanism behind the accumulation of DPPIV on the surface of NPC1 cells, we assumed that endocytosis event in this disease might be delayed due to membrane lipid imbalances resulting in a hindered internalization of proteins which undergo endocytosis hence leading to their accumulation at the cell surface. Polarized protein trafficking has been studied in depth in epithelial (21) and hepatic cells (22), and this allowed the description of DPPIV internalization in these cells as it occurs first through early and then into late endosomes which would later mediate the recycling of DPPIV into the microvilli (21). The mode of entry of DPPIV into fibroblasts has not been described, so first it was necessary to test whether DPPIV itself undergoes endocytosis in fibroblasts. For this purpose, an immunofluorescence endocytosis assay was performed in which two sets of WT and NPC1 cells were kept on ice to block endocytosis, after that one group of cells was transferred to a 37 °C incubator to reintroduce endocytosis whereas the other group was kept on ice. This was followed by immunofluorescent labeling of both groups of cells with an antibody against DPPIV and another one against early endosome antigen 1 (EEA1). The appearance of yellow vesicular structures resulting from colabeling of DPPIV and EEA1 (a typical early endosomal marker) in the cells that were incubated at 37°C indicates that these two proteins colocalize inside early endosomes during the process of entry of DPPIV inside the cells (fig3, A).

Endocytosis may occur via various mechanisms. Among the clathrin-independent mechanism, 'lipid rafts' dependant such as caveolae and flotillin-mediated pathways have been described in the literature (23, 24, 25). A biotin endocytosis assay was conducted to compare the difference between endocytosis of DPPIV in WT and NPC1 fibroblasts. In this assay, proteins on the cell surface were labeled with a cleavable biotin analogue (NHS-SS-biotin) on ice. Internalization of the appropriate biotin tagged cell surface proteins was initiated by returning the surface biotinylated cells to a temperature of 37°C at different time points. Cells were then treated with glutathione which cleaved the disulfide linkage hence removing the NHS-SS-biotin label from non-internalized cell surface proteins, whereas internalized biotinylated proteins were protected from this cleavage. These proteins were later immunobloted with streptavidin-HRP. The appearance of biotin-labeled protein that is resistant to glutathione reduction was taken as an indicator of internalization. In (fig 3, B) it appears that the DPPIV band corresponding to 20 min and to 2 hours of incubation at 37 ºC is less in NPC1 cells than in WT cells by almost 2-folds corresponding to the 2-fold increase of this protein on the cell surface and indicating a possible slow-down in endocytosis.

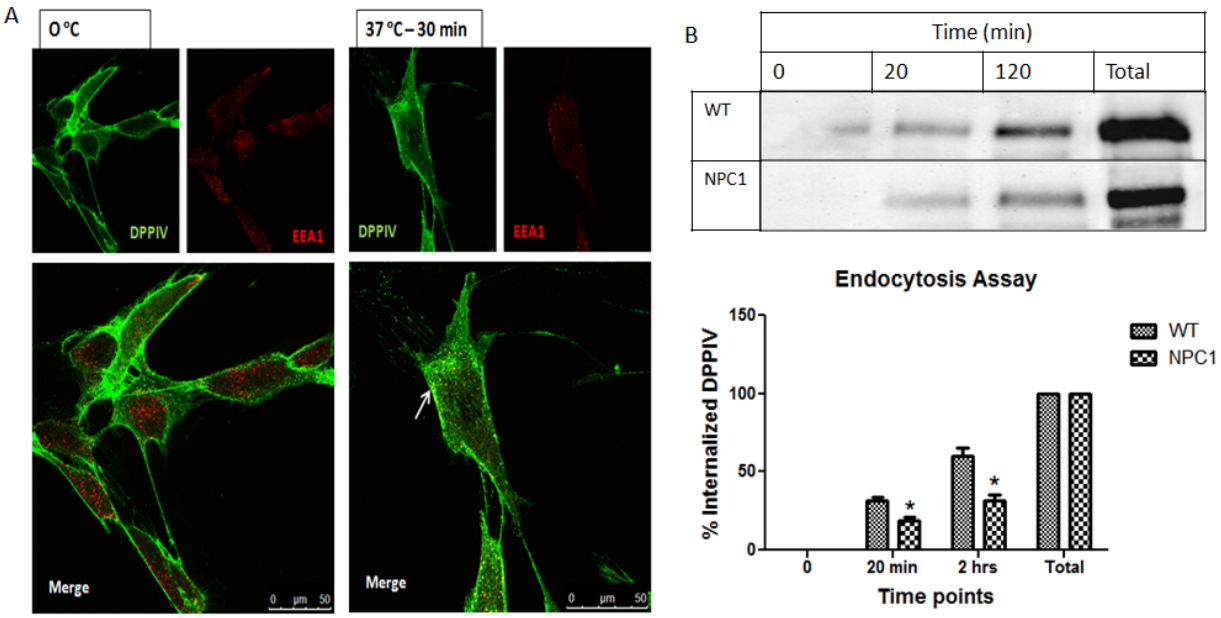

Figure 3 Delayed endocytosis of DPPIV in NPC1 cells. A) To determine the mode of DPPIV entry into fibroblasts, immunostaining was performed using antibody against DPPIV (green) and EEA1 (red). Some cells were kept on ice whereas others were put back in 37ºC for 30 min. The colocalization between the two proteins is depicted in the yellow vesicular structures appearing in the cells that were incubated at 37 ºC indicates that DPPIV enters the cells through endocytosis. B) A biotin endocytosis assay was conducted for WT and NPC1 (patient1) cells. In this assay, biotin was used to label proteins on the surface at 4°C. Surface biotinylated cells were returned to a temperature of 37°C at different time points to allow the internalization of the appropriate cell surface proteins with their biotin tag. Quantification of the endocytosis assay results revealed that after 2hrs 28% of DPPIV was internalized in NPC1 cells compared to 56% in WT cells, indicating a two folds delay in endocytosis of DPPIV in NPC1 cells. (* $p < 0.05$, $n \geq 3$ independent experiments).

N-butyldeoxynojirimycin (NB-DNJ) inhibits glucosylceramide-synthetase and by this it blocks the first step in glycolipid synthesis and thus prevents their progressive accumulation. This substance is commercially available for the treatment of Niemann-Pick type C (NPC). Here, NPC1 cells were incubated with various concentration of NB-DNJ and above-mentioned abnormalities were analyzed again. Treatment of NPC cells with NB-DNJ resulted in an apparent restoration of lipid rafts shown by the shift of flotillin2 back into the floating fractions of the

gradients (fig 4, A). By doing FACS analysis the level of DPPIV on the cell surface of NPC1 cells appeared to gradually decrease with increasing concentrations of NB-DNJ (fig 4, B) reaching a level similar to WT when incubated with 200µM of this drug. When NPC1 cells were incubated with NB-DNJ, the internalized DPPIV bands appeared to intensify signifying an enhancement in the endocytosis of this protein as revealed by the quantification of these blots (fig 4, C). Thus, it could be shown that NB-DNJ reverses the abnormalities found in the fibroblasts derived from the NPC patient.

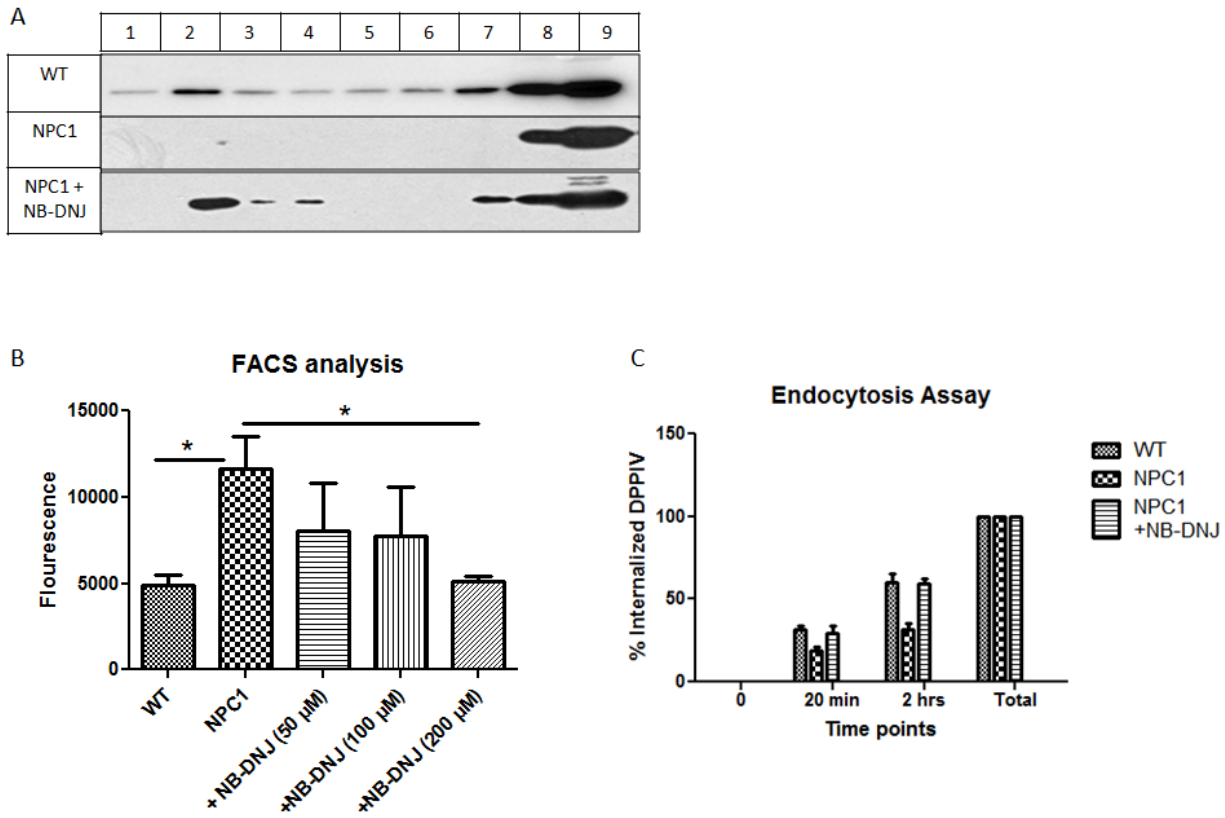

Figure 4 Incubation of NPC1 cells with NB-DNJ rescues the present biochemical abnormalities. A) The distribution of the 'lipid raft' associated protein flotillin2 was shown to be altered as described above. The same procedure was repeated for NPC1 (patient 1) cells treated with NB-DNJ, and the result was an apparent restoration of lipid rafts shown by the shift of flotillin2 back into the floating fractions of the gradients (fractions 1 to 4). Representative immunoblot from one out of four WT cells is shown. B) NPC1 (patient 1) cells were incubated with several concentrations of NB-DNJ and cell surface DPPIV was

analyzed with FACS analysis, where the mean value ± SEM of four normal WT fibroblasts is shown. C) Biotin endocytosis assay was performed in the presence of NB-DNJ (The mean value ± SEM of two experiments is shown). The delayed endocytosis of DPPIV mentioned above was reversed when the NPC1(patient1) cells were incubated with NB-DNJ where the internalization of DPPIV goes back to levels close to normal. (* $p < 0.05$, n=3 independent experiments).

In conclusion, the findings of this study assume that the mechanism behind the accumulation of 'lipid raft' associated protein DPPIV on the cell surface of NPC1 is a result of the impaired 'lipid rafts' composition which in turn leads to a delay in the endocytosis event. Previously, defective endocytosis was reported in NPC1 null cells that were found to have altered localization of lipid rafts associated protein annexin 2 (26), and defective endo/lysosomal trafficking was found in NPC1 cells when fluorescently labelled lactosylceramide (BODIPYLacCer) appeared to accumulate in endosomes and lysosomes of peripheral blood B lymphocytes derived from an NPC1 patient (27). Interestingly, in both of these studies when GSL synthesis was inhibited, the phenotypes relating to impaired endocytic trafficking were both relatively reversed. Lloyd-Evans et al. clearly stated in 2008 that calcium signaling is disrupted in NPC1 disease as a result sphingosine accumulation, and since calcium is important for normal endolysosomal trafficking its altered homeostasis leads to the accumulation of cholesterol and sphingolipids (28). These studies do point out to impaired endocytosis as a characteristic phenotype in NPC1 disease. However, this study shows for the first time that certain proteins build up on the cell membrane as a result of a defective endocytosis event. It should be noted here that another NPC1 patient was examined for cell surface DPPIV and for flotillin 2 distribution. This second patient had a heterozygote mutation for p.I1061T and p.H1016L. The disease of this patient was characterized by a late infantile onset with classical

NPC course. Regarding cell surface DPPIV and flotillin 2 distribution in this second patient, the results showed a similar pattern to what was obtained in the first patient. Results are shown in supplementary material (Figure S1). This opens some possibilities of using DPPIV or any other lipid rafts associated protein that might have a trafficking defect as potential biomarkers for NPC1. NB-DNJ, by inhibiting GSL synthesis and restoring some balance in the membrane lipid composition, was able to repair the lipid rafts and allow proper endocytic event to take place hence rescuing these biochemical abnormalities observed in NPC1 cells.

Supplementary material

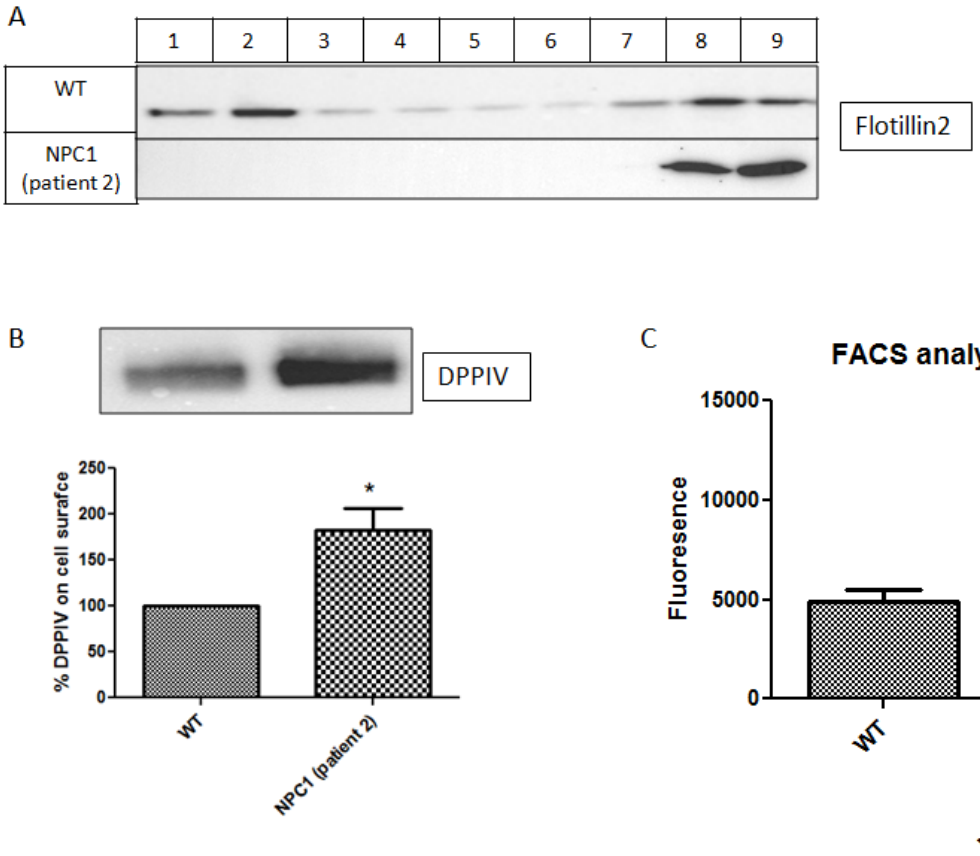

Figure S1. Patient 2 had a heterozygote mutation for p.I1061T and p.H1016L. This patient had a late infantile onset with classical NPC course.

A) The distribution of the 'lipid raft' associated protein flotillin2 was shown to be altered in patient 2 by performing density based sucrose gradients for triton x-100 DRMs, which revealed a significant decrease of this 'lipid raft' protein from the floating fractions (1 to 4) and its accumulation in the soluble fractions (fractions 8 and 9). B) biotin-avidin system based assay was performed and isolated cell surface proteins were then immunoblotted with a monoclonal DPPIV antibody. DPPIV was around twofold increased on the cells surface of NPC1 (patient 2) cells relative to WT. A representative immunoblot with only one of four WT cells is shown C) FACS analysis was also performed, a twofold increase of this protein was observed on the membranes of NPC1 (patient 2) cells relative to the mean value ± SEM of four normal WT fibroblasts. (* $p < 0.05$, $n \geq 3$ independent experiments).

Acknowledgements

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Chapter 5

Discussion

5. Discussion

In this study the aim was to investigate biochemical pathways involved in the pathogenesis of lysosomal storage diseases (LSDs). Two major diseases were put in focus. M. Fabry disease, an X-linked LSD caused by deficiency of α-galactosidase A (AGAL) which results in an abnormal accumulation of glycosphingolipids (1), and M. Niemann–Pick type C (NPC1) a rare, usually fatal, autosomal, recessive, neurovisceral, LSD caused by mutation in the NPC1 protein which is responsible for intracellular cholesterol trafficking and eventually results in accumulation of cholesterol and glycosphingolipids inside lysosomes (2).

The eukaryotic cell membrane consists of a rather complex composition of various lipids and proteins. The massive amount of research in the past decades that has been directed to study biological membranes arrived to the agreement that the organizational structure of cell membrane consists mainly of a lipid bilayer which segregates into a bulk of glycerophospholipids, and microdomains enriched in sphingolipids and cholesterol along with their associated proteins. There is now general agreement that these microdomains known as the “lipid rafts” play major regulatory functions in cellular processes particularly in signaling and trafficking pathways (3).

The fact that LSD pathogenesis and lipid rafts are correlated is hardly surprising especially in LSDs involving raft lipids such as sphingolipidoses (M. Fabry , Gaucher, Tay-Sachs, Sanfhoff and others) and LSDs with cholesterol trafficking defect like NPC1. An imbalance in the composition of a specific cluster will eventually lead to a defect in the function associated with that microdomain.

5.1 M. Fabry and lipid rafts

The defective alpha-galactosidase activity in Fabry disease is responsible for the accumulation of glycosphingolipids, primarily globotriaosylceramide (Gb3), also known as ceramidetrihexoside, in addition to galabiosylceramide and blood group B and P glycosphingolipids.

In a study done previously in our lab on fibroblasts from patients with M. Fabry, lipid analysis revealed that sphingomyelin (SM) levels were elevated in Triton X-100 DRMs, and the overall glycolipid content in these Fabry fibroblasts was approximately threefold more than in fibroblasts from healthy individuals. Knowing that the trafficking of many proteins occurs in association with such microdomains, we decided to look at a DRM associated protein Dipeptidyl peptidase IV (DPPIV) in Fabry fibrobalsts (4). DPPIV has been used several times by our group as a model for studying protein trafficking in health and disease and was previously shown in intestinal epithelial cells to be associated with cholesterol and sphingolipid rich microdomains (5). The trafficking of DPPIV appeared to be hampered in Fabry fibroblasts whereas that of the non-DRM associated Aminopeptidase N was not affected (4). In a follow up on this study we analyzed lipid rafts in four Fabry fibroblasts by isolating Triton-X 100 DRMs and running density based gradients. Flotillin 2 which was used as a 'lipid rafts' marker protein appeared in Fabry fibroblasts to have an altered distribution within the fractions of the sucrose density gradient, as it shifted from the floating fractions towards the soluble fractions of the gradient reflecting a possible change in structure of the microdomain to which it belongs. On the other hand, non–lipid rafts associated protein RhoA appeared in both Fabry and WT cells in the soluble fractions of the gradient. The same Fabry fibrobalsts were then incubated with agalsidase alfa as enzyme

replacement therapy and N-butyl-deoxynojirimycin (NB-DNJ) an inhibitor of glucosylceramide synthase as substrate reduction therapy. As a result, flotillin 2 shifted back into the floating fractions of the gradient which indicates that by reducing cellular storage material, the balance in lipid rafts structures could be restored and hence the impaired trafficking of associated protein is corrected.

Previously we were able to show that secondary dysfunction of mitochondrial energy metabolism does also occur in fibroblasts from FD-patients as indicated by the decrease in cellular levels of ADP and creatinephosphate (6). The respiratory chain complexes I, II, III, and IV are embedded in the inner mitochondrial membrane and therefore could be directly affected by changes in membrane lipid composition. (1). Whether this decreased activity of several mitochondrial respiratoty chain complexes results from compromised mitochondrial membrane integrity due to “lipid raft” alterations is still under investigation.

In one study, caveolar membranes extracted from α-GAL A knock-out mouse aortic endothelial cells (MAECs) were found to have an age-dependant elevated levels of Gb3, glucosylceramide, and lactosylceramide compared to wild type cells. In turn caveolar proteins (caveolin 1 and 2 and annexin II) were found to have altered levels in these microdomains from α-GAL A knock-out MAECs (7). To better understand the role of glycosphingolipids (GSLs) in caveolar structure formation, and to associate that with the results that were previously observed in α-GAL A knock-out cells, the same group then did a follow up study where they treated endothelial cells with various GSL inhibitors. This resulted in a significant decrease in high molecular weight (>400kDa) caveolin 1 oligomers, but not in smaller oligomers (250 kDa) or caveolin 1 monomers, indicating that GSLs play a role in high order caveolar structures (8).

5.2 NPC1 and lipid rafts

In Niemann-Pick type C1 (NPC1) disease, LDL-derived free cholesterol and sphingolipids accumulate inside the lysosomes due to a defect in NPC1 protein. NPC1 is a large transmembrane protein localized in the endo/lysosomal system and its function is still unclear. There has been recent evidence that sphingosine is the first lipid to accumulate after a defect in NPC1 protein is introduced followed by cholesterol, sphingomyelin and glycosphingolipids (2). This has modified the previous consensus model that sphingolipids are a "secondary" storage phenotype. Regardless of which is the initiating lipid, the fact is NPC1 is cholesterol-sphingolipid storage disorder.

In fibroblasts from a patient with genetically proven NPC1 disease, we checked for lipid raft abnormalities and potentially associated alterations in cellular mechanisms. Sucrose density gradient was performed to separate TX-100 isolated DRMs. Fractions of the gradient were immunoblotted with an antibody against flotillin 2 a "lipid rafts" marker protein. In fibroblasts derived from healthy individuals denoted as wild type (WT) cells, flotillin 2 appears floating in the upper fractions of the gradient (a feature due to buoyant density of lipid rafts). However, in NPC1 fibroblasts a significant reduction in flotillin 2 from the floating fractions in parallel with its accumulation in the soluble fractions of the gradient was seen. This indicates that in NPC1 cells and due to cholesterol-sphingolipid imbalances, the lipid rafts were distorted and have lost their floating trait. In cells lacking a functional NPC1 protein, the arrival of LDL-derived cholesterol to the plasma membrane was defective (9). Therefore, not enough cholesterol is recycled to the membrane to form abundant clustering in the lipid rafts.

RhoA (a non-lipid raft associated protein) appeared in both WT and NPC1 cells in the soluble fractions of the gradient.

Next we decided to examine whether the trafficking of some proteins which are associated with lipid rafts is affected in these NPC1 cells. Again DPPIV was the protein of choice due to the reasons described before. DPPIV was measured by isolating cells surface proteins via a biotin-avidin system assay and doing a western blot with an antibody against DPPIV. The result was a significant twofold increase in the levels of DPPIV on the cells surface of NPC1 cells as compared to WT cells.

To understand the mechanism behind the accumulation of DPPIV on the cell surface of NPC1 cells, we postulated that perhaps endocytosis was impaired. Due to the known role played by lipid rafts in endocytosis (10), an alteration in lipid rafts as seen by the redistribution of flotillin 2 in the density gradient, is very likely to cause a defect in endocytic events. Indeed this was the case, as we performed a biotin-based enocytosis assay which showed that internalization of DPPIV was twofold slower in NPC1 as compared to WT cells. Previously, defective endocytosis was reported in NPC1 null cells that were found to have altered localization of lipid rafts associated protein annexin 2 (11).

In fact it was postulated earlier that there might be a limit to the amount of lipid rafts tolerated by endo/lysosomal system. Therefore an accumulation in either one might cause jamming and hampering of sphingolipid degradation or entrapment of cholesterol in late endosomes (10). This was followed by several reports that lipid rafts were impaired in NPC1 cells (9, 12). The traffic jam model proposed by Simons (2000) could eventually affect other

lipids and proteins and their associated functions such as: mistargeting of raft components, altering membrane properties, defective sorting/trafficking, lysosome biogenesis and autophagy, and deregulated immune response (10). In addition, both sphingolipids and cholesterol are involved in neuronal function, which is where the pathology of NPC and most sphingolipidoses is manifested. (13). Considering all these lipid raft associated mechanisms that are affected by a misbalance in one or both types of raft lipids, might explain the wide variety of clinical features implicated in most LSDs.

Interestingly when production of glycolipids was inhibited by N-butyldeoxynojirimycin (NB-DNJ) (OGT 918, miglustat, Zavesca®manufactured by Actelion) which inhibits glucosylceramide-synthetase and is used as a form of SRT, the observed biochemical abnormalities were rescued. Flotillin 2 reappeared in the lipid raft fractions, and the high DPPIV levels were reduced on the cells surface of NPC1 cells. In addition, NB-DNJ incubated cells were able to show DPPIV internalization kinetics close to normal. These results indicate that by inhibiting production of GLs in NPC1 cells, a cholesterol-glycolipid raft balance is restored to the membranes thus leading to amelioration of storage phenomena.

5.3 Other LSDs and lipid rafts

In a cell model of Gaucher Disease (GD) - an LSD characterized by the accumulation of glucosylceramide (GC) due to a defective β-glucosidase (14) - lipid analysis revealed a dramatic increase in GC and other GSLs concentrations particularly in the DRM regions of the membrane, and this was proposed to be the grounds for the altered lipid and protein sorting in Gaucher disease (15).

Altered distribution of lipid rafts has been also reported in Sandhoff B cells (16) and in Niemann-Pick type A T cells (17).

A common mechanism for altered trafficking in ten sphingolipid storage diseases (SLSDs) (Fabry's disease, GM_1 gangliosidosis, GM_2 gangliosidosis (Tay-Sachs and Sandhoff forms), metachromatic leucodystrophy, mucolipidosis type IV, Niemann-Pick disease (types A, B, and C), and sphingolipid-activator-protein-precursor (prosaposin) deficiency) was reported. Mistargeting of a fluorescent analogue of the glycosphingolipid lactosylceramide (BODIPY-LacCer) to the endo/lysosomes was seen in these SLSDs instead of being trafficked to the Golgi as is the case in normal cells (18). Another common factor among numerous SLSDs was the altered intracellular distribution of cholesterol and NPC1 protein. Interestingly, when cholesterol levels were depleted from SLSD cells, BODIPY-LacCer was normally trafficked to the Golgi, and when normal cells were overloaded with cholesterol mistargeting of BODIPY-LacCer occurred again (19). These results again highlights the reciprocal relation between cholesterol and sphingolipids rafts that is manifested in various LSDs involving these types of lipids.

Overall Discussion and Outlook

Currently there is no cure for any LSD, and diagnosis of many of these rare diseases remains challenging. The clinical spectrum in most LSDs is highly variable, while some patients die at early age, others don't exhibit symptoms until adulthood. Fabry patients are initially screened by enzyme assay, but females usually have a milder manifestation of the disease and may still show a close to normal alpha-galactosidase activity. In the case of NPC1, the primary diagnosis tool is filipin staining, which also shows variable parameters among patients with different mutations. Most of the times diagnosis by biochemical means is insufficient and mutation analysis must be performed from patient fibroblasts. Clearly the need for identifying specific biomarkers for each disease is desperately needed.

The work done in this project and in the other above cited studies shows that lipid rafts are a major contributing factor in LSDs pathogenesis. Lipid rafts organize a range of cellular processes including signal transduction and protein trafficking. DRMs distribution was found altered in several cholesterol-sphingolipid storage diseases, which apparently resulted in altered trafficking of a variety of lipid raft associated proteins such as DPPIV and flotillin 2 that were used as markers in this study. Such proteins could in the future provide helpful tools for diagnosis and monitoring of different types of LSDs. However, a variety of tests still needs to be performed before we arrive to this decisive conclusion about lipid raft proteins.

In the case of NPC1, DPPIV accumulation needs to be verified in other cell types such as lymphoblasts which provide an easier diagnostic tool relative to fibroblasts which require taking a biopsy from patients. To check whether the phenotypes observed in the case of NPC1 are due

to cholesterol or glycolipid imbalance, inhibitors like cyclodextrin or fumonisin could be applied to normal cells, and the previous implicated phenotypes could be assessed.

Further in depth understanding of mechanisms involved in LSD pathogenesis in relation to lipid raft alterations is essential. One important additional step would be doing protein arrays which could identify further downstream biochemical pathways affected by lipid raft alterations in LSDs.

In the end, understanding biochemical pathways involved in the progression of LSD pathogenesis allows the identification of specific implicated proteins or molecules that could be used as biomarkers or targets for therapy.

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EDUCATION

PhD in Molecular (Biochemistry)	Leibniz University Hannover-LUH Hannover, Germany	Expected Sept, 2012
Master of Science (Molecular Biology)	Lebanese American University-LAU Byblos, Lebanon	June,2008
Bachelor of Science (Biology)	Lebanese American University-LAU Byblos, Lebanon	June,2005
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WORK EXPERIENCES

- **Research position as PhD student**

Hannover Medical School – Hannover, Germany *Sept, 2009-Sept, 2012*

Worked on two lysosomal storage diseases (Niemann Pick type C and Morbus Fabry), where I tried to identify pathophysiological pathways involved in these diseases with emphasis on membrane structure and function. The study also involved screening for possible biomarkers and testing the efficiency of current available drugs in restoring some of the biochemical anomalies that we identified in these diseases. Methods used involved western blotting,

cloning, hpTLC, isolation of lipid rafts via sucrose density gradients, quantitative PCR, biotin based cell surface protein isolation and endocytosis assays, and flow cytometry.

- **Research Assistant**

Lebanese American University – Byblos, Lebanon *Aug, 2008 - Aug, 2009*

Worked on several research projects which included techniques such as: Cell Culture, Flow Cytometry, RT-PCR, Western blot and others.

- **Lab Instructor**

Lebanese American University – Byblos, Lebanon *Fall, 2008*

Taught the Cell and Molecular lab at LAU.

- **Food Safety Consultant**

CONCIEL - Bekaa, Lebanon *June, 2007- June, 2008*

Implemented ISO 22000 for Gardenia, a food plant.

- **Chemistry and Biology teacher**

Modern St. Anthony school – Mastita, Lebanon *Sep, 2005 - June, 2007*

Taught chemistry and biology for intermediate and secondary classes.

WORKSHOPS

Lysosomes Sept 29-Oct1, 2011

University Medical Center Hamburg-Eppendorf

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Bone manifestations of Gaucher disease Jan,2011

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Bioinformatics Workshop July,2009

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ISO 22000:2005 Training module – "How to locate CCP's" July, 2007

QUALEB-Beirut,Lebanon

ISO 22000:2005 Training Course – "Internal Audit" July, 2007

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PUBLICATIONS

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Rizk S, Maalouf K and Baydoun E. The Antiproliferative and Pro-apoptotic Effect of Kefir on HUT-102 Malignant T-lymphocytes. Clinical Lymphoma and Myeloma. 2009

Prizes and awards

- Recipient of “third place” poster award in the “Annual Poster Conference in Biology and Biomedical Sciences”, Lebanese American University (LAU) Byblos, Lebanon, May 2008.

- Recipient of full scholarship to pursue my masters studies at the Lebanese American University (LAU), Byblos, 2006-2008.

- Listed on distinction list in the final semester of my Bachelor studies at the Lebanese American University (LAU), Byblos, 2005.

COMPUTER SKILLS

MS Word, Excel, Power Point, ImageJ, GraphPad, Internet applications, Java programming.

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Traveling, camping, music, and swimming are among the activities that I enjoy.

Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried

Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.
Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel:

LIPID RAFTS IMPLICATIONS IN THE PATHOPHYSIOLOGY OF LYSOSOMAL STORAGE DISEASES

selbständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

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(Unterschrift)

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