UNIVERSITÀ DI PARMA

Dottorato di Ricerca in Scienze Medico-Veterinarie

Ciclo XIX

# Characterization of a new animal model of lysosomal storage disease: Gangliosidosis in a family of boars

## Caratterizzazione di un nuovo modello animale di patologia da accumulo lisosomiale: Gangliosidosi in una famiglia di cinghiali


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# Summary

# Abstract

Lysosomal storage diseases (LSDs) are heterogeneous group of rare, progressive, lethal, multisystemic diseases with autosomal recessive inheritance. LSDs are characterized by deficient function of specific lysosomal enzymes, due to a genetic defect. According to the strictly sequential lysosomal degradation pathway of glycosphingolipids, defects for almost every step in their degradation have been described; this lead to a block of degradation and accumulation of the corresponding undegraded substrates. These pathologies can be classified on the base of the underlying genetical defect, the affected enzyme or according to the accumulated products. Gangliosides are major components of neuronal membranes and participate in crucial processes of the nervous system. Genetic defects in catabolism of these glycosphingolipids and their accumulation cause a neuronal LSD, defined Gangliosidosis. These pathologies can be subclassified into GM1 gangliosidosis, caused by β-galactosidase deficiency with GM1 ganglioside accumulation, or GM2 gangliosidosis, caused by β-hexosaminidase or GM2 activator protein deficiency with GM2 ganglioside accumulation. Since the human diseases are relatively rare, animal models are indispensable tools for further study of pathogenesis and for development of potential treatments.

The aim of the project is to study morphological and biochemical features of GM2 gangliosidosis in 3 wild boars of the same brood. Three littermate (3/3) wild boards, from a free ranging farm, presented neurological signs (dysmetria, ataxia, quadriplegia and lateral decubitus) at 6 month of age. Viral and bacterial analysis were performed and resulted negative for bacteria culture, Classical Swine Fever and Aujeszky; plants and drugs toxicosis were excluded. Due to the worsening conditions, they were euthanized at approximately one year of age and submitted for necropsy. Gross lesions, common in all affected animals, were reduction in consistency

of cerebral and cerebellar parenchyma, diffuse hepatic degeneration and gastrointestinal dilation. Histology revealed in brain, cerebellum, spinal cord, peripheral ganglia and retina, enlarged foamy neurons, with diffusely severely vacuolated cytoplasm. Other neuropathological findings included spheroids, meganeurites and microgliosis. Astrocytosis was confirmed with Immunohistochemistry for GFAP and Vimentin; demyelination was evident with Luxol fast blue stain. Electron Microscopy analysis of neurons revealed that cytoplasm was enlarged by the presence of numerous lysosomes, singularly disposed or in aggregates, filled by membranous material arranged in lamellae and whorls (membranous cytoplasmic bodies). Biochemical studies revealed the presence of an elevate amount of GM2 ganglioside, confirming the diagnosis of GM2 gangliosidosis.

GM2 gangliosidosis in swine has been described only in 1978 in purebred Yorkshire swine (*Sus scrofa domestica*) and has never been described in wild boars (*Sus scrofa*). This form of GM2 gangliosidosis in a family of wild boars is very similar to human disease and can be a potential useful animal model.

# Introduzione

Le patologie da accumulo lisosomiale (LSDs) sono un gruppo eterogeneo di rare, progressive, letali, multisistemiche patologie con un meccanismo ereditario autosomico recessivo. Le LSDs sono caratterizzate da una funzionalità deficitaria di enzimi lisosomiali specifici, causata da difetti genetici. A partire dalla via di degradazione lisosomiale strettamente sequenziale degli sfingolipidi, sono stati descritti difetti per quasi tutti i passaggi della loro degradazione; ciò determina un blocco metabolico e un accumulo di substrati corrispondenti non degradati. Queste patologie possono essere classificate secondo il difetto genetico, l'enzima deficitario o i prodotti accumulati. I gangliosidi sono uno dei maggiori componenti delle membrane neuronali e partecipano a processi cruciali per il sistema nervoso. Difetti genetici nel catabolismo dei gangliosidi ed il loro accumulo determinano una LSD neuronale, definita Gangliosidosi. Questa patologia può essere sottoclassificata in Gangliosidosi GM1, causata da una deficienza di β-galattosidasi con accumulo di ganglioside GM1, o Gangliosidosi GM2, causata da deficienza di β-esosaminidasi o proteina attivatrice GM2 con accumulo di ganglioside GM2. Poiché la patologia nell'uomo è relativamente rara, i modelli animali sono strumenti indispensabili per studi sulla patogenesi e per lo sviluppo di potenziali trattamenti.

Lo scopo del progetto è di studiare le caratteristiche morfologiche e biochimiche di un caso di Gangliosidosi GM2 in tre cinghiali della stessa nidiata. I tre cinghiali, appartenenti ad un allevamento brado, hanno presentato sintomatologia neurologica (dismetria, atassia, quadriplegia e decubito laterale) a 6 mesi di età. Sono state eseguite analisi virologiche e batteriologiche, risultate negative per culture batteriche, Peste suina classica e Aujeszky; sono state inoltre escluse tossicosi indotte da piante e farmaci. A causa del peggioramento delle condizioni cliniche, i cinghiali sono stati sacrificati a circa un anno di età e sottoposti a indagini necroscopiche. Lesioni macroscopiche, comuni in tutti gli animali affetti, erano riduzione della consistenza del

parenchima cerebrale e cerebellare, degenerazione epatica diffusa e dilatazione gastrointestinale. L'esame istologico ha rilevato nel cervello, cervelletto, midollo spinale, gangli periferici e retina, la presenza di neuroni aumentati di dimensioni, con citoplasma diffusamente e severamente vacuolizzato. Altre lesioni neuropatologiche includevano sferoidi, meganeuriti e microgliosi. I fenomeni di astrocitosi sono stati confermati con colorazioni immunoistochimiche per GFAP e vimentina; mentre la colorazione Luxol fast blu ha mostrato diffusi quadri di demielinizzazione. La microscopia elettronica ha evidenziato nel citoplasma dei neuroni la presenza di numerosi lisosomi, disposti singolarmente o in aggregati, ripieni di materiale membranoso organizzato in lamelle o vortici (corpi citoplasmatici membranosi). Studi biochimici hanno rivelato la presenza a livello cerebrale di un'elevata quantità di ganglioside GM2, confermando la diagnosi di Gangliosidosi GM2.

Questa patologia fu descritta nel suino solamente nel 1978 nella razza Yorkshire (*Sus scrofa domestica*) e non è mai stata descritta in cinghiali selvatici (*Sus scrofa*). Questa forma di Gangliosidosi GM2, evidenziata per la prima volta in una nidiata di cinghiali, appare molto simile alla patologia riscontrata nell'uomo e può rappresentare un utile modello animale.

# 1. Lysosomes and Sphingolipids

## 1.1. Lysosomes

Lysosomes are membrane-bound organelles which maintain a specialized biochemical environment within the cell. They possess a number of lysosomal membrane proteins, including proton pumps, which actively maintain an acidic pH which is the optimum working environment for 40 different acid hydrolases (best function in pH $\leq$ 5); these hydrolases include proteases, nucleases, lipases, glycosidases, phosphatases, and sulfatases.

Lysosomal enzymes are initially synthesized in the ER lumen and then tagged with a mannose-6-phosphate (M6P) residue within the Golgi apparatus. Such M6P-modified proteins are subsequently delivered to lysosomes through trans-Golgi vesicles that express M6P receptors. These enzymes break down a variety of different, mostly large, molecules, hence lysosomes have historically been thought of as the “recycling centres” of the cell. Macromolecules destined for catabolism in the lysosomes arrive by one of three other pathways:

- Material internalized by *fluid-phase pinocytosis* or *receptor-mediated endocytosis* passes from plasma membrane to early endosome to late endosome, and ultimately into the lysosome. The early endosome is the first acidic compartment encountered, while proteolytic enzymes only begin significant digestion in the late endosome; late endosomes mature into lysosomes. During the maturation process, the organelle becomes progressively more acidic.
- Senescent organelles and large, denatured protein complexes are shuttled into lysosomes by a process called *autophagy*. Through poorly understood mechanisms, obsolete organelles are corralled by a double membrane derived from the endoplasmic reticulum; the membrane progressively expands to encircle a collection of structures and forms an *autophagosome* which then fuses with lysosomes and the contents are

catabolized. In addition to facilitating the turn-over of aged and defunct structures, autophagy is also used to preserve cell viability during nutrient depletion.

- *Phagocytosis* of microorganisms or large fragments of matrix or debris occurs primarily in professional phagocytes (macrophages or neutrophils). The material is engulfed to form a *phagosome* that subsequently fuses with a lysosome. [1]

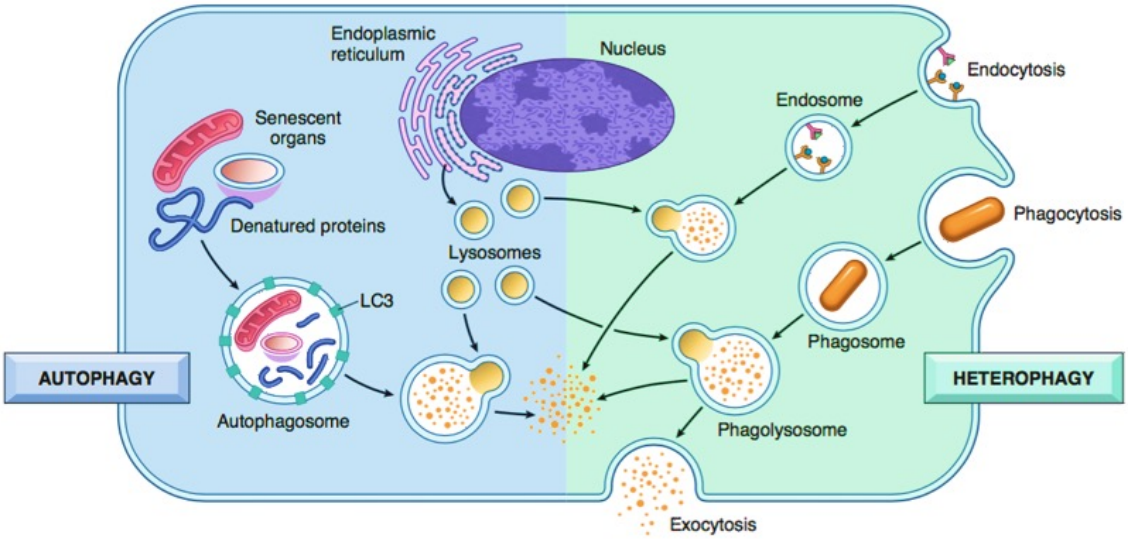



*Fig. 1 Schematic representation of lysosomal degradation. Comparison between heterofagy (right side) and autophagy (left side). [1]*

Lysosomes are not simply “end organelles” but a central metabolic coordinator in highly complex regulatory and recycling mechanisms essential for normal cell function. New functions and properties of the lysosomes continue to be discovered each year including calcium storage, intracellular trafficking, paracrine functions and the regulation of autophagy. [1] [2]

There is a dynamic inter-action between the endosomal and lysosomal system involving the secretory–endosomal–autophagic–lysosomal–exocytic network, which is needed for proper processing of cellular macromolecules. Many cellular

events regulate lysosome function, including pH, calcium homeostasis, nutrient surveillance through amino acid sensing, and starvation response through mTOR localization and modulation. Activation of lysosomal function eliminates damaged proteins through chaperone-mediated autophagy, eliminates misfolded proteins and aggregates through macro-autophagy, maintains mitochondrial homeostasis through mitophagy, induces phagocytosis and receptor-mediated endocytosis, and regulates its own biogenesis function upon activation of the transcription factor EB (TFEB) and transcription factor E3. TFEB has been found to regulate a specific gene network of approximately 500 genes involved in lysosomal biogenesis and autophagy, referred to as the coordinated lysosomal expression and regulation network [3]

## 1.2. Sphingolipids

### 1.2.1. Structure and function

Together with glycerophospholipids and cholesterol, sphingolipids are building blocks of eukaryotic membranes. They are characterized by the presence of a sphingoid base within the hydrophobic part of the molecule. In sphingomyelin and the glycosphingolipids, a phosphorylcholine or a carbohydrate moiety are bound to the terminal hydroxyl group of ceramide (N-acylsphingosine), respectively. [4]

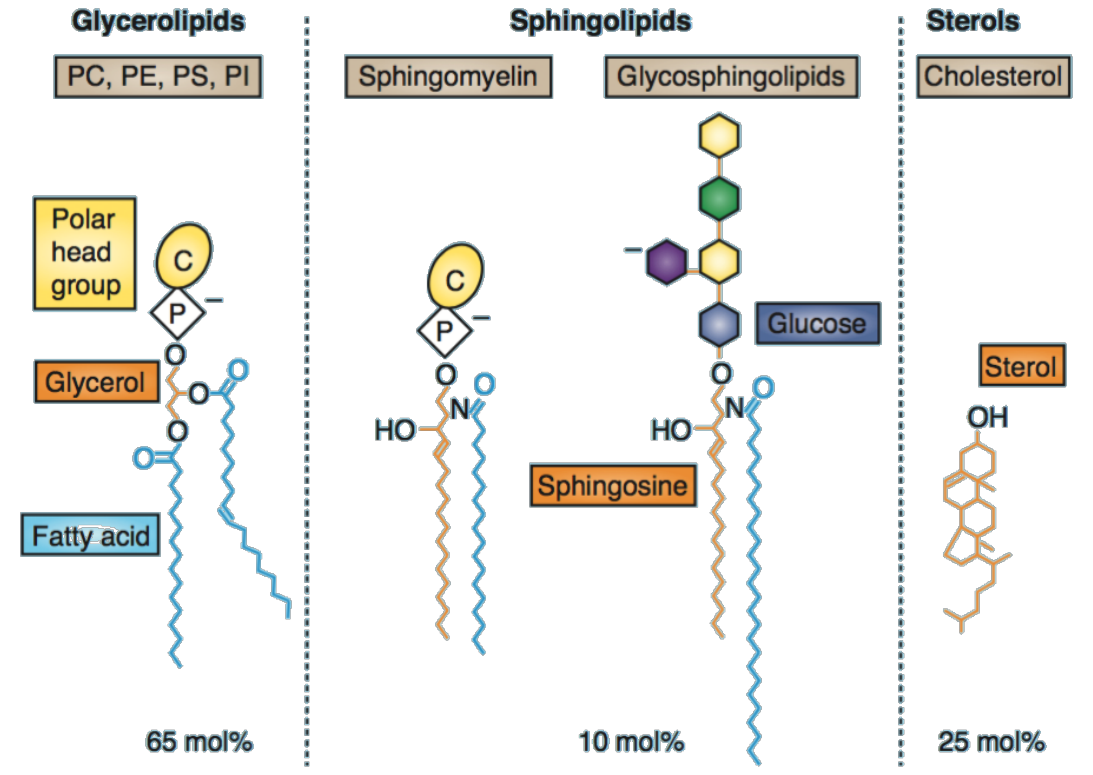



*Fig. 2 Main components of cell membranes [5]*

Glycosphingolipids display a high structural diversity and can be classified into series, which are characteristic for a group of evolutionary related organisms. On cellular surfaces, glycosphingolipids form characteristic patterns and they are believed to segregate into pre-existing microdomains, which are enriched in

glycosyl-phosphatidylinositol-anchored proteins, sphingomyelin, and cholesterol. These so-called lipid rafts might constitute the physiological surroundings of many membrane proteins. [4]. The lifetime of rafts as determined in living cells by STED microscopy is very short, and the upper limit for their diameter is about 20 nm and appear to be only transient structures. [6]

Glycosphingolipids are species- and cell-type specific [6]. Particularly Neuronal plasma membranes (PMs) are enriched and stabilized by gangliosides, sphingomyelin and cholesterol. Gangliosides are major components of neuronal membranes, where they contribute up to 10–12% of the total lipid content and participate in crucial processes of the nervous system. Their regulatory roles are suggested by the dramatic change in the pattern of gangliosides expressed during development of the nervous system, as well as by the region-specific distribution of gangliosides with different carbon chain length in the ceramide moiety. The brain content of sphingomyelin, ceramide and glycosphingolipids (GSLs) changes through-out life, during aging and in neurodegenerative diseases. [7]

The exact biological role of the highly complex ganglioside patterns is unclear, but two different modes of interactions can be assumed as the basis for glycosphingolipid function in general:

1) Glycosphingolipids can mediate recognition events with water-soluble or membrane-bound lectins outside the cell. [6].
2) Glycosphingolipids can interact with proteins present in the same membrane. They can modify the activity of relevant receptors like that for insulin, epidermal growth factor, or nerve growth factor. [4]

There are convincing evidences that various SL molecules are bioactive lipids regulating numerous physiological functions, including neural cell apoptosis, differentiation and repair, dendritogenesis and neurotransmitter release. [8] In addition to disorders caused by alterations of sphingolipid metabolism, these

are involved in a variety of diseases. In infectious diseases, sphingolipids serve, e.g., as pathogen receptors and can control pathogen infection and host defense. In the immune system, glycosphingolipids play a role as antigens (ABO-System, Forssman), but can also stimulate the generation of autoantibodies in post-infectious autoimmune diseases, like Guillain–Barré or Miller–Fisher Syndromes. They are ligands for CD1d-restricted natural killer T-cells (NKT-cells); for example, the highly immunogenic, non-human α-galactosylphytoceramide activates a subset of NKT cells. [4]

### 1.2.2. Biosynthesis

Sphingolipids can be formed by de novo-biosynthesis from L-serine and fatty acids, or by the recycling of building blocks within the so-called “salvage” pathway. [6]

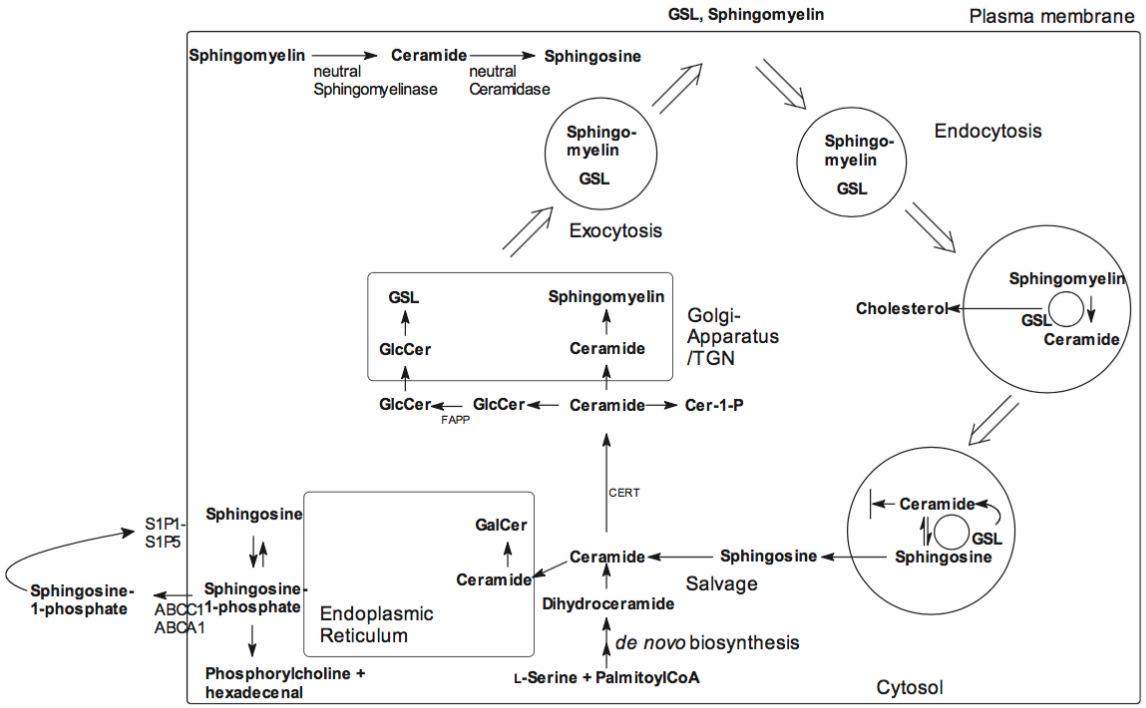



*Fig. 3 Schematic representation of sphingolipid metabolism [6]*

Salvage can be the by far prevailing process in some cell types, especially in differentiated cells like neurons. The steps leading to ceramide formation take place at the cytoplasmic leaflet of the membrane of the Endoplasmic Reticulum.

The first step of sphingolipid de novo-biosynthesis is catalyzed by serine palmitoyltransferase (SPT), an enzyme that is composed of three subunits, SPTLC1, SPTLC2 (catalytic subunit), and SPTLC3. The pyridoxalphosphate-dependent condensation of l-serine and coenzyme A-thioesters of saturated fatty acid, preferentially palmitic acid, leads to the formation of 3-ketosphinganine. This is rapidly reduced by the enzyme ketosphinganine reductase to sphinganine. Acylation of sphinganine to dihydroceramides with different acyl chain lengths – but also of sphingosine that arises only from sphingolipid degradation – is carried out by acyltransferases encoded by lass-genes (homologues of the yeast longevity assurance gene lag1). Mammals contain six different acyltransferases that display specificities for coenzyme A-thioesters of different fatty acids. The lass-genes are expressed in a cell-type specific fashion, so that different cell types form ceramides of different acyl chains. Dihydroceramides can be desaturated to ceramides, or oxidized at the 4-position of the sphinganine moiety to phytoceramides. Sphingosine-1-phosphate is a primary and second messenger that can be formed in response to extracellular stimuli like interferon or tumor necrosis factor. It is formed by sphingosine phosphorylation mediated by sphingosine kinases 1 or 2, and can then be transported out of the cell by the members of the ABC-transporter family ABCC1 and ABCA1. As an autocrinic and paracrinic regulator, it binds to the G-protein-coupled receptors S1P1-S1P5 on cell surfaces. S1P antagonizes ceramide-mediated effects, acts as a mitogen and inhibits apoptosis, and regulates calcium ion homeostasis and cell growth. The sphingosine1-phosphate signal is terminated by phosphatase-mediated cleavage of the phosphate moiety, or by a lyase-meditated, pyridoxal phosphate-dependent cleavage of the sphingoid backbone between C2 and C3. A less well characterized mediator lipid is sphingosylphosphorylcholine. Also, ceramides can be formed in response to intracellular and extracellular stimuli and mediate mostly antimitogenic effects such as cell differentiation and apoptosis. In higher concentrations, they are found in the human skin, where they are required for

the water permeability barrier. In differentiated keratinocytes, skin ceramides differ in their structure from those found in other tissues and can contain very long acyl chains. These can be hydroxylated in 2-postion, but can also be hydroxylated and acylated especially with linoleic acid to form acylceramides. Skin ceramides are transported in form of glucosylceramides and sphingomyelins, which are released from lamellar bodies to the interface of the stratum granulosum and stratum corneum of the skin, where they are cleaved by endolysosomal hydrolases. Skin ceramides can also be covalently bound to proteins of the cornified envelope of the stratum corneum and are the precursors of protein-bound fatty acids. Head group attachment to ceramide occurs at different sub-cellular sites: in cells that express galactosylceramide synthase, galactosylceramide (GalCer) is formed in the lumen of the ER, and glucosylceramide (GlcCer), the precursor of most glycosphingolipid series, is formed at the cytoplasmic face of the Golgi membrane. Ceramide-1-phosphate, a lipid with proliferative and pro-survival properties is formed by ceramide kinase, which localizes to the cytoplasmic face of the Golgi apparatus and other intracellular membranes. Sphingomyelin synthesis is catalyzed by sphingomyelin synthase 1 in the lumen of the trans Golgi, or by sphingomyelin synthase 2at the plasma membrane. From the ER, ceramide is transported to its destination either via vesicular flow, or mediated by the transport protein CERT. Glc-Cer can reach the cytoplasmic face of other cellular membranes, eventually by transport with the glycolipid transfer protein, or is carried to its site of transversal translocation to the anticytoplasmic face of intracellular membranes by the protein FAPP2 (four-phosphate adaptor protein 2). This happens either at the trans Golgi or the ER. Neither the site of GlcCer and GalCer translocation nor the identity of the required translocator protein is unambiguously known. The candidate proteins include the ABC transporter ABCA12 that is deficient in Harlequin ichthyosis and might transport GlcCer through the endosomal or plasma membrane. Transmembrane glycosyltransferases including sialyltransferases with their active sites in the

lumen of the Golgi apparatus catalyze the formation of higher glycosphingolipids including gangliosides from GlcCer. Biosynthesis of more complex glycosphingolipids continues by the stepwise addition of single carbohydrate residues. In the biosynthesis of gangliosides, lactosylceramide and gangliosides GM3, GD3, and GT3 serve as precursors for complex gangliosides, which can bear no (0-series), one (a-series), two (b-series), or three (c-series) sialic acid residues linked to the inner galactose moiety. The transferases that catalyze early glycosylation steps show high specificity towards their glycolipid substrate, while those acting more down-stream are less specific. As a consequence of glycosyltransferase topology, glycosphingolipids with the exception of the cytoplasmic oriented GlcCer pool face the anticytoplasmic face of cellular membranes and contribute to the glycocalix of cellular surfaces. During embryonic development, different glycosphingolipid series are expressed, for example, brain development is accompanied by a shift from simple gangliosides like GM3 and GD3 to more complex ones like GD1a and GT1b during synaptogenesis and myelination. The initially proposed biosynthetic scheme was confirmed by lipid analysis of the mutant animals. The phenotypes of knockout these mice indicate a vital role of gangliosides for reproduction, neuronal function, and insulin action. For example, double mutant mice deficient in the sialyltransferase and N-acetylgalactosaminyltransferase that act on ganglioside GM3 express only GM3 as their major ganglioside instead of a complex ganglioside pattern, but in about the same amount. These animals are extremely susceptible to sound stimuli that produced lethal seizures and display a sudden death phenotype. 0-series glycosphingolipids, which are usually not observed in vivo, occur in mice deficient in the sialyltransferase GM3-synthase. In the kidney, ganglio series glycosphingolipids can bear sulfate residues instead of sialic acids. The steps leading to their formation have also been investigated with knockout mice that have defects in ganglioside biosynthesis. Complex gangliosides that occur in mouse testes additionally contain fucose residues and polyunsaturated very long acyl chains. Their deficiency might

account for infertility observed in some of the knockout models. [6]

In most cell types, de novo-biosynthesis constitutes only a minor pathway for sphingolipid formation; metabolic labelling studies indicate that the majority of glycosphingolipids are formed by the recycling of building blocks within a salvage-pathway. After their biosynthesis, glycosphingolipids reach the plasma membrane through vesicular exocytotic membrane flow. [4]

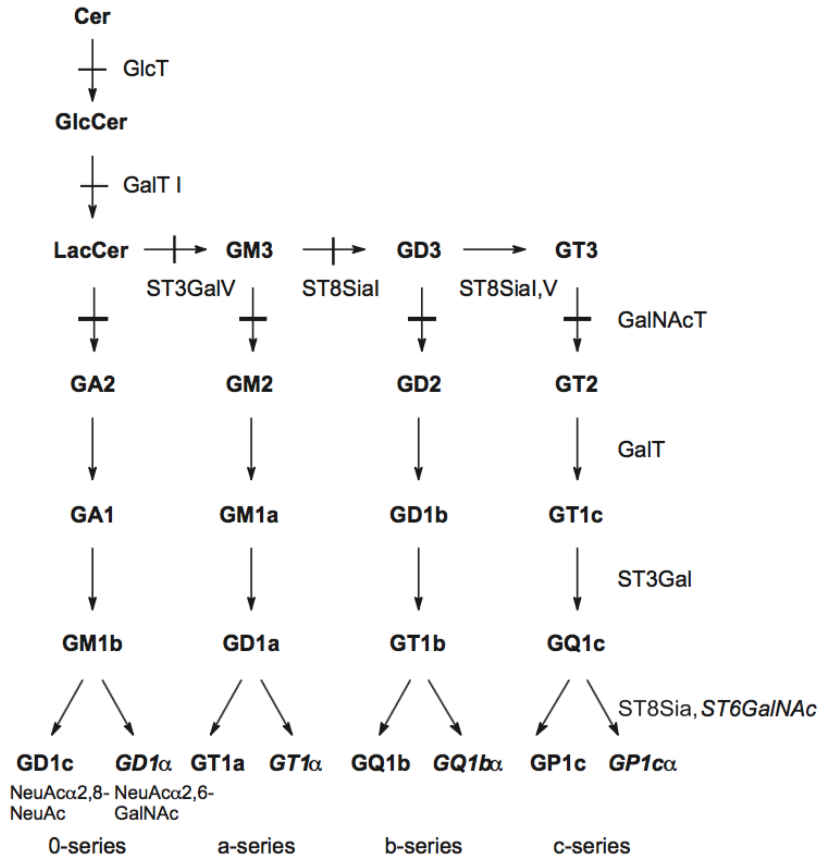



*Fig. 4 Schematic representation of ganglioside biosynthesis [6]*

### 1.2.3. Degradation

Constitutive degradation of (glyco)sphingolipids takes place in endosomes and lysosomes. Together with other membrane components, most (glyco)sphingolipids enter the acidic compartment via endocytosis and are cleaved into their building blocks at the surface of intraendosomal and intralysosomal membranes. Degradation of these membranes and lipid

aggregates requires the presence of hydrolytic enzymes, lipid transfer proteins, and bis(monoacylglycero)phosphate as a negatively charged lipid, that is formed and exclusively found on these luminal membranes. Adjustment of the lipid composition of intraendosomal membranes is part of this finely tuned process, such as the degradation of sphingomyelin to ceramide by acid sphingomyelinase, and the NPC2-mediated exit of cholesterol. [9]

(Glyco)sphingolipid degradation is a stepwise process, so that deficiency of one single step causes accumulation of the substrates in the endolysosomal compartment. Due to the lipid nature of the storage material, this cannot leave the compartment. Different human diseases are known that are caused by the deficiency of lysosomal enzymes and lipid transfer proteins, but also of nonlysosomal proteins that are required for this process. [6]


*Fig. 5 Schematic representation of lysosomal degradation of sphingolipids and selected glycosphingolipids [6]*

In addition to endolysosomal degradation, sphingolipids are also cleaved in other compartments e.g. by cermidases and sphingomyelinases. A nonlysosomal-glucosidase that has been implicated in the hydrolysis of bile acid glucosides cleaves also gluco-sylceramides. Its deficiency in mice leads to male infertility. [6]

#### 1.2.3.1. Topology of endocytosis and lysosomal degradation

Located in the outer (exoplasmic) leaflet of the plasma membrane, sphingolipids are degraded within the process of endosomal/lysosomal membrane digestion. Together with other macromolecules and mem- brane components they are transferred into the lysosomal compartment by autophagy, phagocytosis and endocytosis.

At the level of endosomes, sphingolipids reach luminal intraendosomal vesicles or intraendosomal membranes for digestion; these intraendolysosomal vesicles are generated during endocytosis by successive steps of vesicle budding and fission controlled by the endosomal sorting complex required for transport (ESCRT). They are platforms for lipid and membrane degradation as demonstrated in prosaposin deficient cells. We assume that luminal vesicles or IMs are prepared for lysosomal digestion by a lipid sorting process beginning at the level of endosomes.

Studies suggest that at least subpopulations of exosomes, intraluminal vesicles, which can be secreted after fusing with the plasma membrane, are formed in an ESCRT independent pathway.

Membrane-stabilizing cholesterol is sorted out mainly by two sterol binding proteins, NPC-2 and NPC-1. In vitro, cholesterol of liposomal membranes inhibits lipid solubilization by Sap-A and –B. NPC-1 is a transmembrane protein of the endosomal perimeter membrane, whereas NPC-2 is a small soluble gly- coprotein. NPC-2 might remove cholesterol from IM and deliver it to NPC-1 which exports the lipid from endosomes. In vitro, the cholesterol transfer between liposomes, mimicking endolysosomal vesicles, is strongly inhibited by sphingomyelin (SM). Degrading liposomal SM with acid sphingomyelinase

releases the inhibition. This might explain that in Niemann–Pick disease types A and B, patients deficient of acid sphingomyelinase accumulate not only SM but also large amounts of cholesterol. During endosomal maturation the pH decreases and the degradation resistant anionic lipid bis(monoacylglycero)phosphate (BMP) is formed from phosphatidylglycerol in the IM. In vitro BMP stimulates cholesterol transfer between liposomes and sphingolipid degradation substantially.

In contrast to the luminal vesicles, the lysosomal limiting membrane is protected on the inner leaflet by a thick glycocalix containing hardly digestable polylactosamine structures. The glycocalix is formed by highly N-glycosylated integral membrane proteins, and stabilized by high cholesterol levels, and chaperone HSP70. The high lateral pressure of the limiting membrane presumably also attenuates the insertion of the GM2 activator protein (GM2-AP), a lipid binding protein essential for ganglioside catabolism. [10]

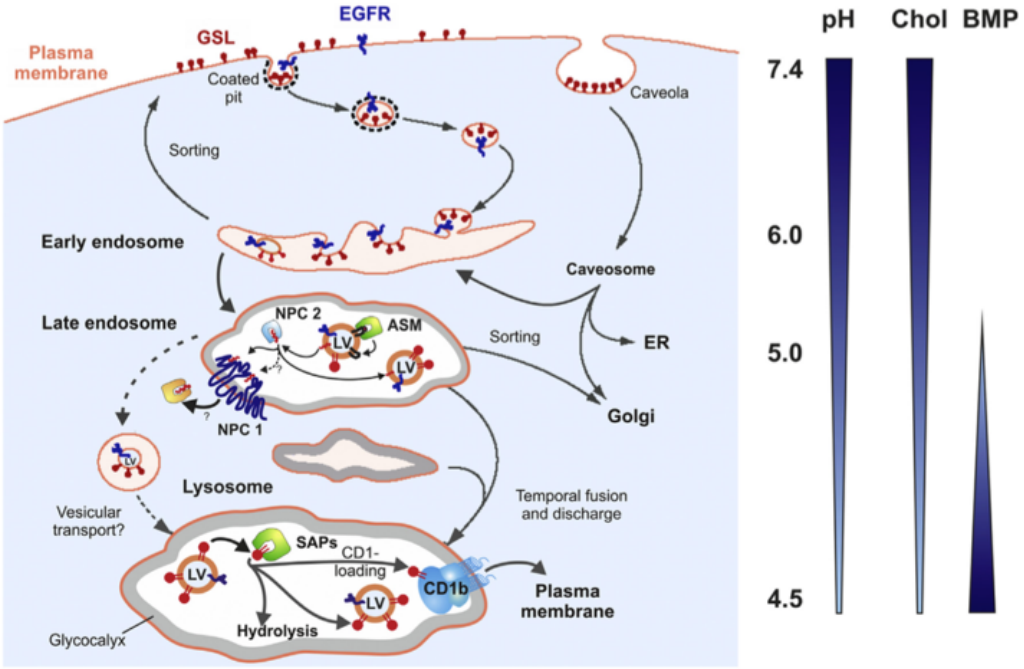



*Fig. 6 Schematic representation of proposed topology of endocytosis and lysosomal degradations. Values of pH, cholesterol and bisphosphates [11]*

### 1.2.3.2. Sphingolipid activator proteins

Monosaccharide residues from the non-reducing end of the oligosaccharide part of the glycosphingolipids are sequentially cleaved off by the action of acid exohydrolases. The substrates of these enzymes are embedded in intraendosomal and intralysosomal membranes, whereas the enzymes are dissolved in the lysosol. In vivo, glycosphingolipids with less than four sugar residues are only degraded in the presence of sphingolipid activator proteins (SAPs). SAPs mediate the interaction between the membrane bound lipid substrate and the water-soluble enzyme or activate the enzyme directly. In vivo, enzymatic hydrolysis of most membrane-bound sphingolipids is also stimulated by anionic lysosomal lipids, especially by bis-(monoacylglycero)-phosphate (BMP, lysobisphosphatidic acid), which concentrates in the inner membranes of lysosomes. Many catabolic reactions in this pathway require the presence of an activator protein. In vitro, these SAPs can often be replaced by detergents.

To date, five sphingolipid activator proteins (SAPs) are known: the GM2 activator protein and the four saposins (Saps) -A, -B, -C, and -D. The GM2-activator is an essential cofactor in the in-vivo degradation of ganglioside GM2 by β- hexosaminidase A; the inherited deficiency of this protein leads to the AB variant of GM2 gangliosidoses [4]

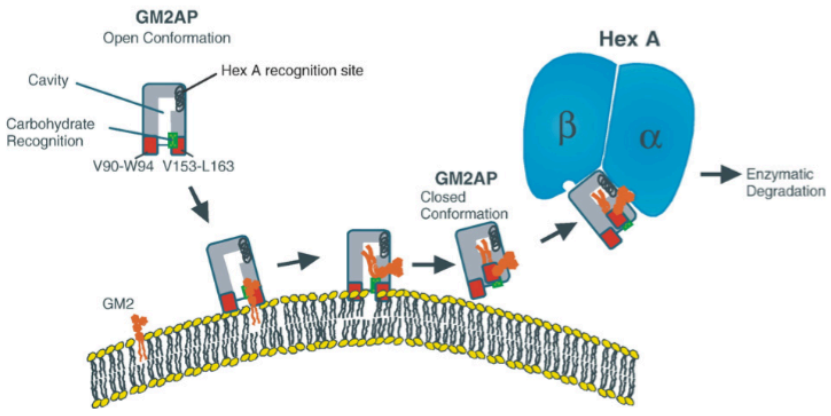



*Fig. 7 Schematic representation of function of GM2 activator [4]*

For the presentation of ganglioside GM2 or related glycosphingolipids, e.g., GM1, to the active site of the degrading enzyme, the GM2-activator has to insert into the bilayer of intralysosomal lipid vesicles. The lipid recognition site of the activator interacts with the substrate, so that the hydrophobic ceramide portion of the substrate becomes embedded in the hydrophobic cavity of the activator protein. After a conformational change of the lipid-loaded activator increasing the water-solubility of the complex, this is released from the membrane, and ganglioside GM2 is presented to the enzyme and is subsequentially degraded. The Saps or saposins A–D are glycoproteins with molecular weights of 8–11 kDa. They belong to a family of saposin-like proteins with lipid binding and membrane perturbing properties. Although the four Saps share a high degree of homology and some properties, they act differentially and show different specificity. This leads to the different phenotypes of the different Sap deficiencies. Besides their function as enzyme cofactors, sphingolipid activator proteins play an important role in lipid antigen presentation. They participate in the loading of lipid antigens to immunoreceptors such as human CD1b, human, and mouse CD1d. [4]

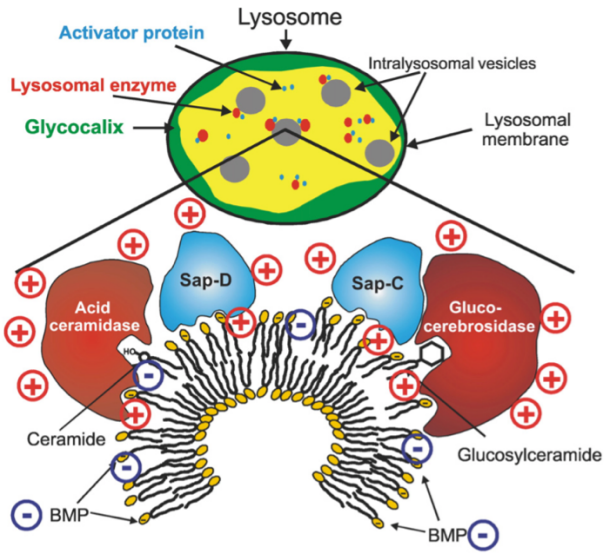



*Fig. 8 Schematic representation of sphingolipid degradation at membrane-water interface of luminal lysosomal vescicles* [10]

### 1.2.3.3. Degradation of gangliosides

Degradation of gangliosides occurs at the surface of endolysosomal vesicles and IM, rich in anionic lipids like BMP. Soluble enzymes and membrane perturbing lipid binding SAPs with isoelectric points above the surrounding lysosomal pH values are positively charged. The protonated cationic amphiphilic proteins can therefore bind to the anionic, BMP rich membrane surface.

Cationic amphiphilic drugs such as desipramine reach the IM in the lysosomal compartment and compensate their anionic surface charge. They interfere with the electrostatic binding of lysosomal proteins to the anionic surface of IM, and attenuate the lysosomal catabolism. Cationic amphiphilic drugs release lysosomal proteins from the IM surface and trigger their proteolysis, e.g. that of ASM. [10]

# 2. Inherited diseases in lysosomal glycosphingolipid degradation

The sphingolipidoses are a group of inherited diseases, which are caused by defects in genes encoding proteins involved in the lysosomal degradation of sphingolipids. Together with mucopolysaccharidoses, mucolipidoses, glycoprotein, and glycogen storage diseases, sphingolipidoses belong to the lysosomal storage diseases (LSDs). [4]

LSDs can occur due to:

- A deficiency of one of the hydrolytic enzymes contained in the lysosomes.
- A deficiency of one of the hydrolytic enzymes cofactors (such as sphingolipid activator proteins).
- A deficiency of one of the lysosomal membrane proteins (e.g. transporters).
- Defective intracellular trafficking (transport systems involved in the degradation process)
- Defective activation of lysosomal enzymes. [2]

The diseases are usually named according to the identity of the storage material or later focused on deficient enzyme or cofactor. Because different genetic mutations may cause varying degrees of a specific hydrolase deficiency, variants of a particularly biochemical form of disease may occur. These variants are sub-classified on clinical grounds by descriptive terms such as early or late onset, type I/II, type A/B, or by the precise molecular defect present. [12] According to the strictly sequential degradation pathway of glycosphingolipids in humans, defects for almost every step in the degradation of these lipids have been described [4] and about 50 genetically different forms are actually known. [2] With exception of the 2 X-linked diseases in humans (Fabry disease [α-

galactosidase deficiency], MPSII [iduronate sulfatase deficiency]) and possibly one human form of adult onset ceroid-lipofuscinoses, all inherited lysosomal storage diseases have autosomal recessive traits. Domestic animals tend to occur in breeds, usually developed in specific localized from a small number of founders and associated with inbreeding to fix type. Thus, breeds tend to be genetically less diverse than human population and occurrence of a genetic disease is likely to reflect a single mutation. Breeding practices, characterized by the use of relatively few sires, line breeding and fashions in selective breeding, may lead to a relatively high prevalence of a disease in large extended families. [12]

With a reported frequency in animals of single cases or rare families and a collective frequency in human of 1 in 7000–100000 live births, the LSDs are rare disorders. [4] The number of patients varies between very few known cases with prosaposin deficiency on the one hand, and Gaucher disease as the most frequent sphingolipidosis. Striking differences in birth prevalences between countries can be observed and these can, indeed, at least partially be explained by differences in immigration patterns or isolation, for instance due to geographical, lingual, ethnic or religious preferences or customs. For example, in Gaucher disease, which occurs in a frequency between 1:40000 and 1:60000 in the general population, and between 1:500 and 1:1000 among the Ashkenazi Jewish population (Sawkar et al., 2006), 95% of the patients suffer from the non-neuropathic type 1 without involvement of the nervous system. [6] the remarkable high birth prevalences of MPS VI, GM1 gangliosidosis and fucosidosis in the United Arab Emirates (UAE) are another example and primarily due to ethnic isolation and founder effects, which is illustrated by the observations that 95% of genotyped patients were homozygous for their LSD causing mutation and that, indeed, most patients were from the same tribes or blood-related [13]

## 2.1. Pathogenesis of Sphingolipidoses

Sphingolipidoses display a high degree of phenotypic variability. Onset, development, and symptoms vary widely between different sphingolipidoses, but can also differ drastically within one and the same disease.

- One primary factor that determines the pathogenesis of the diseases is the cell-type specific pattern of glycosphingolipid expression. Storage in patients occurs especially in those cells and organs in which the substrates of the corresponding deficient degrading system are prevalently synthesized or taken up by phagocytosis.
- the second important factor is the residual activity of the defective enzyme in many, although not all LSDs. Onset and severity of the storage disease is partly determined by the residual activity of the gene product in the lysosomes. A complete deficiency of a lysosomal enzyme leads to an early onset and a severe course of the disease, whereas only a few percent of residual activity can be sufficient to delay the onset of the disease, cause an attenuated course, and lead to the often misdiagnosed adult forms of the diseases. Although this is valid for most of the LSDs, patients with identical genotype and, accordingly, formal similar residual activity, can undergo different courses of a disease. Apparently, other genetic and epigenetic factors contribute to the expression of a disease in an individual patient.
- A direct consequence of the metabolic blockage is the accumulation of the corresponding enzyme substrates, in addition, the pathogenesis can be influenced by the nature of the storage material. The degradation disorder can be accompanied by formation of bioactive substances. A highly cytotoxic substance, galactosylsphingosine (psychosine) is not sufficiently degraded in cells of patients suffering from Krabbe disease. It leads to the destruction of affected cells, before a significant accumulation can occur.

- Secondary to lipid and glycolipid storage, downstream effects of lysosomal storage contribute to the pathogenesis of these diseases. inflammatory responses like macrophage activation or cytokine release have been, but also on lipid trafficking, phospholipid metabolism, calcium ion homeostasis, and mitochondrial function have been observed. [6]

### 2.1.1. Cell type-specific expression of glycosphingolipids

Differences in the organ involvement of sphingolipidoses can be largely attributed to the cell type-specific expression of glycosphingolipid series: those cells and tissues are predominantly affected, where most (glyco)sphingolipids are formed by biosynthesis, or taken up by endocytosis. This implies that manifestation of the diseases can vary drastically between different sphingolipidoses: while gangliosidoses affect predominantly the grey matter of the brain, Krabbe disease and Metachromatic Leukodysdrophy (Gieselmann and Krägeloh-Mann, 2010) affect the white matter of the brain, Fabry disease is especially a disease of heart and kidney, and Farber disease affects the skin. Also within one and the same disease, broad heterogeneities can be observed. Patients display large genotypic and phenotypic heterogeneity: different genotypes can cause similar clinical symptoms in the patients, and the outcome of a disease can be different even among patients with identical mutations. [13]

Complex gangliosides are predominantly formed in neurons and defects in ganglioside degradation lead initially to damage of the central nervous system. Another sphingolipid, ceramide, is essential for skin function and for the formation of the water permeability barrier. Aberrant ceramide formation by glucosylceramide hydrolysis in lamellar bodies accounts for the severe skin-phenotype of collodian babies with a complete loss of glucosylceramide-glucosidase activity. Galactosylceramide and sulfatide are characteristic lipids of myelin, so that in both, Krabbe's disease and Metachromatic Leukodystrophy, the myelin-forming cells are primarily affected. Alternatively, the lipid load of a

cell may not be primarily determined by endogenous synthesis of the respective lipid, rather than by uptake. [6]

### 2.1.2. The threshold theory and enzyme pseudodeficiencies

The phenotypic diversity between patients of a single sphingolipidoses can be largely attributed to the different residual activities of the affected proteins. A complete deficiency of a lysosomal enzyme leads to an early onset and a severe course of the disease, whereas only a few percent of residual activity can be sufficient to delay the onset of the disease and cause an attenuated course. The ratio of substrate influx into the lysosome and the capacity of the degrading system determines the course of the disease. This is treated in quantitative terms by the so-called “threshold theory”. Only the decrease of enzyme activity below the critical threshold value causes storage of the corresponding lipid substrate. Decrease of enzyme activity to the calculated threshold value does not influence the turnover rate of the substrate, and pathological storage occurs only below this level. With the certain exception of acid ceramidase, a decrease of enzyme activity to values of 20% of normal cells, a typical range for heterozygote carriers of inherited diseases, has no impact on the turnover rate *v* [6]

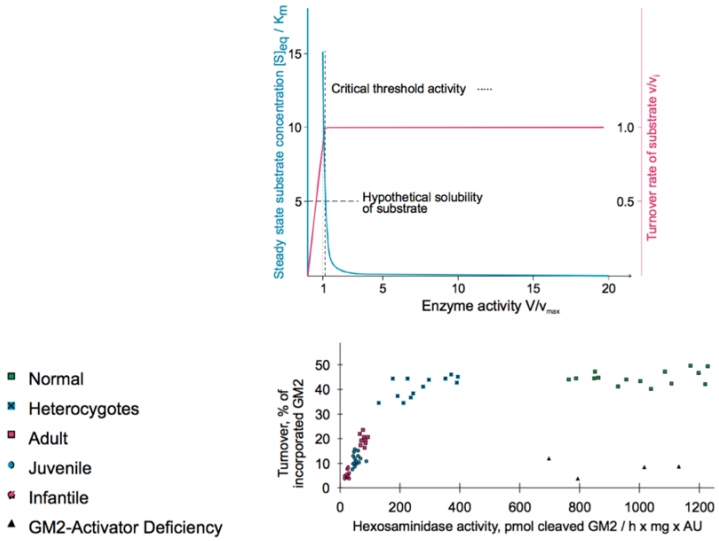



*Fig. 9 Threshold theory. Calculated (top) and experimental (bottom) values for GM2 gangliosidosis. [6]*

The experimentally observed values support the theory since the turnover rate decreases linear with decreasing enzyme activity only below a threshold value. Patients with adult onset of GM2 gangliosidosis show significant higher turnover rates than patients with juvenile course, and these higher turnovers than patients with infantile course. The threshold theory has been experimentally confirmed for the sphingolipidoses, for example GM2-gangliosidoses, Metachromatic Leukodystrophy and Gaucher disease. It is also the quantitative basis for the therapy of sphingolipidoses, since the theory predicts that a small increase of the degradation capacity translates into a significant increase in substrate turnover and improvement of the course of the disease. If substrate influx into the lysosomal compartment is blocked, no storage can occur: double knockout mice with defects in both, ganglioside biosynthesis and degradation, show a late-onset neurological disease due to the accumulation of oligosaccharides, but suffer from a bone disease due to the accumulation of non-degradable glycosaminoglycans. At the same time, this genetically engineered mouse model is the genetic proof of principle for substrate reduction therapy of sphingolipidoses. [6]

For some LSDs, so-called enzyme pseudodeficiencies have been described. This term refers to individuals who have a substantial reduction of enzyme activity, but do not develop any lysosomal accumulation. Pseudodeficiencies are due to polymorphisms in the genes of the respective enzymes that cause the reduction of enzyme activity. [6]

### 2.1.3. Metabolic blockage and properties of the storage substance

The cellular consequences of substrate accumulation are determined by several factors: the type of storage material, the extent of storage, the type of storing cells, and the direct or indirect consequences that lysosomal storage has on basic cellular processes such as intracellular trafficking and autophagy. [14]

Properties of the storage material can have an impact on the pathogenesis of sphingolipidoses. The most prominent example for a specific effect of a storage substance on pathogenesis is Krabbe disease, in which galactosylsphingosine a cytotoxic substance, is not sufficiently degraded. A hallmark in Krabbe disease (globoid-cell leukodystrophy), and also in Metachromatic Leukodystrophy, is the destruction of myelin caused by destruction of myelin-forming cells. Psychosine contributes to the damage especially of oligodendrocytes as the predominantly affected cells due to the cell type-specific expression of gala-series glycosphingolipids. Destruction of cells can occur more rapidly than galactosylceramide accumulation. Apparently, psychosine is always formed by glycosidation of sphingosine as a byproduct of galactosyl-ceramide formation. It is not formed by deacylation of GalCer. In Fabry disease, lysoglobotriaosylceramide is excreted in urine and is discussed as a biomarker in tissues and body fluids. In addition to lyso(glyco)sphingolipids in sphingolipidosis patients, other glycosphingolipids that are positively charged under physiological conditions have been found under normal conditions in neuronal tissues, but their function is largely unknown. Other examples for the formation of toxic substances are glucosylsphingosine in Gaucher disease, lysosulfatide in metachromatic leukodystrophy, sphingosylphosphorylcholine in Niemann–Pick-disease and lysoglycosphingolipids in the other disorders. Neuronal dysfunction might also develop in response to morphologically active substances like ganglioside GM2, which is normally expressed on neurons during neuritogenesis, with subsequent formation of meganeurites and axonal spheroides.

The majority of the amphiphilic storage compounds is not excreted by the affected cells, although it is known that lysosomes of some cell types can secrete their contents after fusion with the plasma membrane, or undergo calcium ion-regulated exocytosis for plasma membrane repair. Extracellular occurrence of storage material, as that of sulfatide in urine and cerebrospinal fluid in metachromatic leukodystrophy, has been attributed to lysosomal

exocytosis. The growing amount of accumulating material might initially lead to a mechanical damage of the cell, and subsequently to apoptosis. Hypertrophy of affected organs is frequently observed, although the storage material itself contributes only to a small extent to mass increase. For example, an increase in heart weight by 1000 g cannot be explained by the accumulation of an amount of 3.5 g globotriaosylceramide as the storage substance. Therefore, hypertrophy secondary to lysosomal storage is an important factor in pathogenesis.

A characteristic feature of sphingolipidoses is the accumulation of other lipids as secondary storage products. These arise from the lipid nature of the primary storage compounds that co-precipitate other hydrophobic material and produce a kind of traffic jam. Niemann–Pick disease, type C, which is due to deficiencies of the endosomal cholesterol export proteins NPC1 or NPC2 with primary cholesterol storage, are named according to the concomitant storage of sphingomyelin, the primary storage substance in types A and B of Niemann–Pick disease. Secondary storage lipids can contribute to the disease phenotype that is not directly correlated with the primary storage substance. For example, secondary accumulation of ceramide and ganglioside GM3 might account for insulin resistance observed in Gaucher disease patients. [6]

In GM2 gangliosidosis, GM2 ganglioside accumulation inhibit the reuptake of calcium into the endoplasmic reticulum by lowering the Vmax of sarco/endoplasmic reticulum Ca++ ATPase (SERCA). SERCA is the endoplasmic reticulum located calcium transporter which pumps calcium from the cytoplasm back into the endoplasmic reticulum to terminate Ca++ mediated cellular responses. Since inhibition of SERCA activity has been shown to result in neuronal apoptosis this may provide at least a partial explanation for neurodegeneration in the GM2 gangliosidoses, Tay–Sachs and Sandhoff disease, respectively. Since secondary accumulation of GM2 ganglioside is a widespread phenomenon in lysosomal storage diseases alterations of SERCA

activity may play a more general role in pathogenesis of lysosomal disorders. [15] [14]

Furthermore, metabolic labelling experiments revealed a decreased level of phospholipids in the neurons from Sandhoff mice, confirmed by in vivo labelling experiments in mice. Labelling of phosphatidycholine was decreased in the brain of GM2 ganglioside storing Sandhoff mice whereas labelling in liver and spleen was unaffected. The same applies also for the steady state levels of these lipids in mice and autopsy samples of patients. Key enzymes in the synthesis of phosphatidylcholine and phosphatidylserine are CTP:phosphocholine cytidylyl-transferase and phosphatidylserine synthase, respectively. Compared to wild type mice, activities of both enzymes are decreased in the brain of Sandhoff mice. A direct inhibitory effect of GM2 ganglioside was excluded. Rather data suggest that regulation of activity of these enzymes occurs posttranslationally. This suggests that GM2 ganglioside storage affects synthesis of phospholipids which are major components of cellular membranes and precursors of important lipid second messengers. [16] [14]

Secondary effects, which are only partially clarified, are induced by the properties of the primary storage substances and contribute to the pathogenesis. These secondary effects include inflammatory responses like macrophage activation or cytokine release as observed in patients of Gaucher disease, in mouse models of gangliosidoses, and in other lysosomal storage diseases. Phospholipid metabolism, calcium ion homeostasis, and mitochondrial function can also be altered. Oxidative stress, unfolded protein response (ER-stress), hypertrophy secondary to lysosomal storage, and alterations of autophagy have also been reported. [6]

# 3. Classification of human sphingolipidoses

The diseases are usually named and classified according to the identity of the storage material. According to the strictly sequential degradation pathway of glycosphingolipids in humans, defects for almost every step in the degradation of these lipids have been described. Most enzymes and cofactors deficient in the sphingolipidoses have been characterized, their genes have been cloned, and animal models of most of the sphingolipidoses have been created by targeted disruption of the respective genes in mice [4]

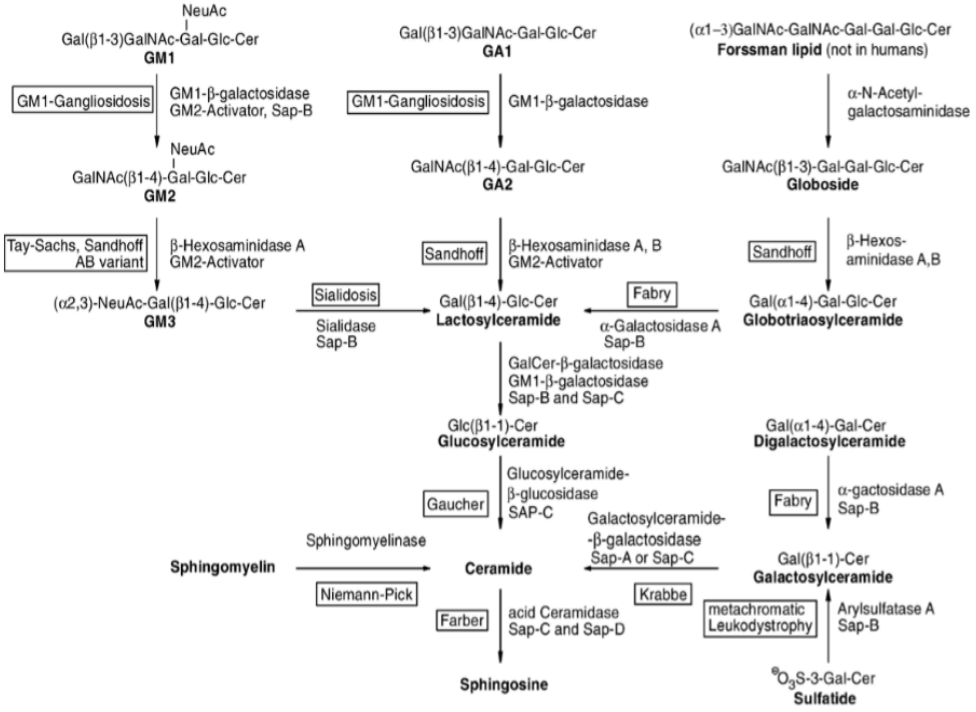



*Fig. 10 Schematic representation of Sphingolipidoses classification (substrates, enzymes, sphingolipid activator proteins and name of diseases) [4]*

## 3.1. GM1-gangliosidosis

GM1-gangliosidosis is caused by an inherited deficiency of the lysosomal enzyme GM1-β-galactosidase. GM1-β-galactosidase is a protein of 64 kDa, which is derived from an 88-kDa precursor. [17] It occurs as part of a lysosomal multienzyme complex, together with sialidase, the so-called protective protein, cathepsin A, and N-acetylaminogalacto-6-sulfate sulfatase. [18] GM1-β-

galactosidase catalyzes the hydrolysis of ganglioside GM1 to GM2 in the presence of either the GM2-activator protein or Sap-B. According to the substrate specificity of the variant enzyme, an inherited defect can also lead to another disorder, Morquio disease, type B. Storage of galactosylceramide-β-galactosidase substrates, galactosylceramide and lactosylceramide, does not occur. [17] Three clinical forms of GM1-gangliosidosis can be distinguished:

- Infantile (type 1): developmental arrest and progressive deterioration of the nervous system occur in early infancy. Characteristic symptoms are exaggerated startle responses to sound, a macular cherry-red spot, hepatosplenomegaly, rigospasticity associated with seizures, and generalized skeletal dysplasia. Most patients die within the first 2 years of life.
- Late infantile/juvenile form (type 2): progressive neurologic symptoms in children
- Adult/ chronic form (type 3): occurs in young adults; extrapyramidal signs frequently presenting as dystonia are the most common neurologic manifestations. Dysmorphic changes are less prominent or absent in these clinical forms. [4]

Apart from the ganglioside GM1, other enzyme substrates also accumulate, such as glycolipid GA1, oligosaccharides from glycoproteins, and intermediates of keratan sulfate degradation. These substances are stored in different organs, according to their major site of biosynthesis. [4] Massive lysosomal GM1-storage in neurons leads to degeneration of the nervous system. On the other hand, formation of meganeurites and ectopic dendrogenesis are presumably due to the antineurotoxic, neuroprotective, and neurorestorative properties of ganglioside GM1 in the plasma membrane. [19] The severity and progression of the disease correlates with the residual enzymatic activity in cells and body fluids. [4]

Gene mutations identified in GM1 gangliosidosis and Morquio B disease include

missense/nonsense mutations, duplications/insertions, and insertions causing splicing defects. Neither the type, nor the location of mutations in the gene of GM1-β-galactosidase could be correlated to the phenotype of the patients. [4] A successful treatment of this disease is not available to date; bone marrow transplantation did not correct the neurological phenotype of the juvenile variant of the disease. [20] A chemical chaperone therapy for the brain pathology has been suggested [21], and substrate reduction therapy reduces storage levels in the brain of the mouse model of the disease. [22]

Morquio type B disease clinically resembles a mild phenotype of Morquio A disease, where keratan sulfate accumulates due to N-acetylgalactosamine-6-sulfatase deficiency. Like GM1-gangliosidosis, Morquio type B is due to the inherited defect of GM1-β-galactosidase. It is characterized by the predominant storage of keratan sulfate and oligosaccharides with terminal galactose residues. Patients show generalized skeletal dysplasia. Involvement of the nervous system and hepatosplenomegaly are absent. The differences between GM1 gangliosidosis and Morquio B disease can be attributed to a lower affinity and activity of β-galactosidase variants towards substrates with Gal-β1,4-GlcNAc motifs in Morquio patients compared to the Gal-β1,3-GalNAc motive present in ganglioside GM1. [4] [23]

## 3.2. GM2-gangliosidoses

The GM2-Gangliosidoses are caused by defects in degradation of ganglioside GM2 and related glycolipids leading to accumulation of these lipids, most fatally in neuronal cells. GM2 is degraded by cleavage of the β-glycosidic linkage between the N-acetylgalactosaminyl residue and the galactose residue by β-hexosaminidases. This reaction requires the GM2 activator protein in vivo. The three lysosomal β-hexosaminidases differ in the combination of their two subunits (α and β), and in their substrate specificity. β-Hexosaminidase A (αβ) cleaves off terminal β-glycosidically linked N-acetylglucosamine and N-acetylgalactosamine residues from negatively charged and uncharged

glycoconjugates by a retaining double-displacement mechanism. The enzyme has two active sites, one on the α-chain, and the other on the β-chain. β-Hexosaminidase B (ββ) predominantly cleaves uncharged substrates like glycolipid GA2 and oligosaccharides with terminal N-acetylhexosamine residues. The crystal structure of human β-hexosaminidase B has been solved and shows the two active sites at the homodimer interface, where most of the alterations due to mutations leading to Sandhoff disease are located. β-Hexosaminidase S (αα) is of secondary significance for GM2 degradation, but it contributes to the degradation of glycosaminoglycans and sulfated glycolipids. [24]

*Tab. 1 Subunit composition of Hexaminidase isoenzymes*

| Hexaminidase Isoenzyme | Subunit composition |
|---|---|
| **A** | αβ |
| **B** | ββ |
| **S** | αα |

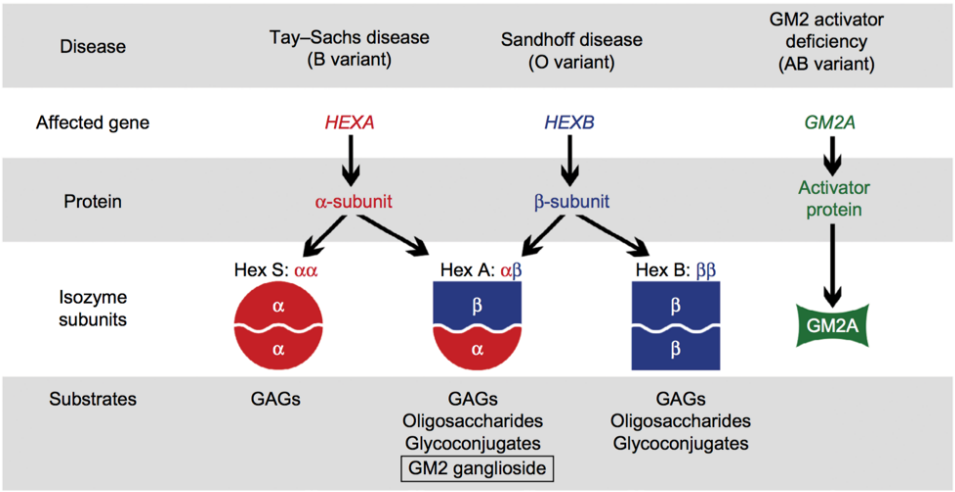
The inborn deficiency of the GM2-activator as well as the deficiency of the α- or β-chain of the β-hexosaminidase isoenzymes leads to one of the three different variants of this disease that are named according to the isoenzyme remaining intact.

- The B-variant is due to an α-chain deficiency, and the subsequent deficiency of hexosaminidases A and S, but with normal hexosaminidase B. Its infantile form is usually called Tay–Sachs disease.
- The 0-variant, or Sandhoff disease, is caused by the deficiency of the β-chain and the resulting deficient activity of β-hexosaminidases A and B, but remaining activity of β-hexosaminidase S.
- The AB-variant is a consequence of mutations in the GM2-activator gene and is characterized by normal activities of β-hexosaminidase A, -B, and

-S towards the natural substrate GM2 in detergent-containing enzyme assays. [4]

The pathogenesis of the GM2-gangliosidoses is not completely understood. Together with other membrane lipids and proteins, ganglioside GM2 accumulates and precipitates within the lysosomes of cells, particularly in neuronal cell bodies. The induction of misconnections in neuronal dendrites and other cellular processes might be one of the causes of the neurological phenotype. Undegraded storage material might reach, to some extent, cellular membranes like that of the Golgi apparatus or the plasma membrane, and alter membrane composition and function. Bone marrow transplantation (BMT) experiments suggest a complex pathogenetic mechanism that may well involve lytic compounds, for example, in the blood circulation and/or cytokines generated in the brain. [24]

*Tab. 2 Diseases, affected genes, isoenzymes subunits and associated substrates in GM2 gangliosidosis [25]*




### 3.2.1. Tay–Sachs disease (TSD)

Clinically, the B-variant of GM2-gangliosidoses can be subclassified into infantile, juvenile, chronic, and adult onset forms.

- Infantile form: higher prevalence among Ashkenazi Jews with a heterozygote frequency of 1:27. Affected children are normal at birth and

show first symptoms, such as mild motor weakness and increased startle reaction between 3 and 6 months of life. Weakness, hypotonia, poor head control, and decreasing attentiveness are observed and visual symptoms appear. A finding, in most cases, is a cherry red spot in the central retina of the patients. Motor, mental and visual abilities decline rapidly after about 10 month of age. Macrocephaly and seizures are common in the second year. Further deteriorations lead to a vegetative state. Death often occurs between the second and fourth year of life.

- Juvenile form: the first motor symptoms are noted between 2 and 6 years of age and death occurs at the age of 10 to 15 years. At the end of the first decade, loss of speech, increasing spasticity, seizures, loss of vision, and progressive dementia are common manifestations. A vegetative state is finally reached and the patients die frequently as a consequence of intercurrent infections.
- Chronic form: clinical onset is between 2 and 5 years of age. Symptoms include abnormalities in gait and posture. With advanced age, distinct neurological symptoms appear. Mental and verbal intelligence as well as sensory modalities remain intact. The patients can reach an age of 40 years.
- Adult form: the symptoms are very heterogeneous. Neurological disorders such as spinal muscular atrophy and psychoses may be mimicked. Nevertheless, intelligence and visual capability are not affected. [4]

The B1-variant of GM2-gangliosidoses differs enzymatically from the B-variant by an altered substrate specificity of the mutated β-hexosaminidase A. While no activity is detected towards the natural substrate ganglioside GM2 and negatively charged synthetic substrates, synthetic uncharged substrates used for diagnosis are cleaved. The function of the α-chain active site is defective, whereas subunit association, enzyme processing, and the activity of the β-chain are not impaired. Homozygous patients with the B1-mutation show the course

of the juvenile disease. However, a late infantile course was seen in compound heterozygotes with a B1 and a null allele. [26]

### 3.2.2. Sandhoff disease (SD)

The 0-variant of GM2-gangliosidosis was the first gangliosidosis for which the underlying enzymatic defect was identified. It is characterized by storage of negatively charged glycolipids characteristic for Tay–Sachs disease, but also by elevation of uncharged glycolipids such as glycolipid GA2 in the brain and globoside in visceral organs. Clinically, the following forms have been reported:

- Infantile: In addition to the clinical and pathological manifestations present in Tay–Sachs disease (infantile B-variant), organomegaly and slight bone deformations may also occur.
- Juvenile: first symptoms such as slurred speech, cerebellar ataxia, and psychomotor retardation appear at 3 to 10 years. Vision is normal, but spasticity increases and mental function deteriorates gradually.
- Adult: The onset of symptoms is delayed to late adult life. The clinical manifestations in this chronic variant are similar to those of Tay–Sachs disease variants of corresponding ages, with the exception of additional accumulation of uncharged enzyme substrates. [4]

### 3.2.3. AB-variant of GM2-gangliosidosis

In this variant, the deficiency of the GM2 activator protein [27] in the presence of normal β-hexosaminidase A, B, and S activity leads to the accumulation of glycolipids GM2 and GA2. The clinical picture resembles that of Tay–Sachs disease with a delayed appearance of symptoms. [4]

## 3.3. Fabry disease

Fabry disease is an inborn deficiency of lysosomal α- galactosidase A. This homodimer of 50 kDa subunits] catalyzes the lysosomal hydrolysis of globotriaosylceramide. Fabry disease is a panethnic, X-chromosomal-linked

inherited disorder with an estimated frequency of 1:117,000 to 1:40,000 birth. Hemizygous males have extensive deposition of globotriaosylceramide in the lysosomes of endothelial, perithelial, and smooth-muscle cells of blood vessels. Many cell types in the heart, kidneys, eyes, cornea, and the autonomous nervous system are also affected. [28]

The first clinical symptoms usually occur during childhood or adolescence. These include severe pain in the extremities, vascular cutaneous lesions, hypohidrosis, and corneal and lenticular opacities. The disease develops to renal, cardiac, and/or cerebral complications, which are the most common causes of death in the 4th or 5th decade of life. A variant of the disease characterized by a milder progression and a primary impairment of the heart muscle has been attributed to enhanced residual activity of more than 5% of normal of the defective enzyme. [4]

The observed symptoms result from accumulating glycolipids in the affected tissues, the blockage of blood vessels, or both simultaneously. In the kidney, the lesions are due to glycosphingolipid accumulation in several cell types, but renal blood vessels are progressively and often extensively involved. On the other hand, in the nervous system vascular involvement is the predominant cause of the affection.

About 180 different mutations causing Fabry disease have been identified including partial gene rearrangements, splice-junction defects, and point mutations. [28]

The treatment of Fabry disease by enzyme replacement therapy using recombinant α-galactosidase A derived from human skin fibroblasts or CHO-cells has been established. A chemical chaperone approach with galactose was successful in a male patient of the cardiac variant of the disease and extensions of this approach using inhibitors have been reported. Deoxygalactonojirimycin treatment of knock-in mice that express the (R301Q) variant of α-galactosidase A led to an increase of enzyme activity in the heart and reduction of globotriaosylceramide storage. [4]

## 3.4. Gaucher disease

Gaucher disease is the most common form of the sphingolipidoses. It is caused by the deficiency of glucosylceramide-β-glucosidase leading to accumulation of glucosylceramide. Glucosylceramide-β-glucosidase, also called glucocerebrosidase, consists of 497 amino acids and has a molecular weight of about 65 kDa in its glycosylated form. It is a lysosomal enzyme that can associate to membranes. The enzyme can be allosterically activated by Sap-C and by negatively charged phospholipids, of which the lysosomal bis(monoacylglycero)phosphate seems to be of physiological relevance. [29] Three different types of Gaucher disease are distinguished:

- Attenuated form, Gaucher disease type I: nonneuropathic course and is the most frequent form of this disease. It has a frequency of 1: 50 000–200 000 births, but which is higher amongst Ashkenazi Jewish population (1:1000). Life expectancies of these patients range between 6 and 80 years.
- Acute form, Gaucher disease type II: is a very rare panethnic disease characterized by the involvement of the nervous system with early onset and a life expectancy of less than two years.
- Subacute or juvenile form, Gaucher disease type III: is an intermediate variant of the other two types, mainly found in the Northern Swedish population. In this case, the neurological symptoms have a later onset and a slower development than in form II; the survival age of the patients is between a few years and four decades. [4]

In all variants, patients may show hepatosplenomegaly, anemia, thrombocytopenia, and bone damage. The severity of these symptoms differs widely, but is inversely correlated with the residual enzyme activity determined in skin fibroblasts of Gaucher patients.

Even if the enzyme activity is reduced in all cell types, the phenotype of type 1 of the disease is predominantly manifested in macrophages of the reticuloendothelial system, since these cells have to degrade large amounts of

glycolipids derived from the phagocytosis of erythrocytes. Due to the stored material, macrophages acquire a typical morphology, which is characterized by the enlargement of the cell and the occurrence of cytoplasmic linear inclusions. Not only the appearance of these so called “Gaucher cells” in the affected tissues, but also the inflammatory response they induce by the release of cytokines, might account for the hypertrophy of the affected organs, for cortical bone loss, and bone marrow disease. Types II and III of the disease that affect the CNS are characterized by a progressive loss of neuronal cells. This might also be caused by accumulation of glucosylsphingosine, which apparently produces neuronal toxicity and might be also able to induce inflammatory responses.

Approximately 200 mutations at the glucosylceramide-β-glucosidase locus have been found in patients with Gaucher disease, four of which account for about 86% of the cases in the Jewish population and for 68% in non-Jewish population. Two cases of Gaucher disease are known, where the cause is the absence of a sphingolipid activator protein, Sap-C. A certain mutation (N370S) impairs the interaction of variant glucosylceramide-β-glucosidase enzyme with Sap-C.

An animal model most resembling the type II form of the disease has been created by targeted disruption of the glucosylceramide-β-glucosidase gene in mice.

Enzyme therapy for the attenuated form of Gaucher disease (type I) is available: it consists of the use of glucosylceramide-β-glucosidase purified from placenta or recombinantly expressed, which has been modified in the carbohydrate part to contain targeting information for the mannose receptor on macrophages. After treatment of the patients, a normalization of the blood parameters, as well as a reduced weight of liver and spleen can be observed. Due to CNS involvement, enzyme replacement is less promising for types 2 and 3 of the disease. Also, bone marrow transplantations have been accomplished, with variable results. A serious problem is the reversal of bone involvement, which has been overcome

by peripheral blood stem cell transplantation in the animal model. [4] [30] [31] [29]

## 3.5. Metachromatic leukodystrophy

Metachromatic Leukodystrophy (MLD) is caused by the inherited deficiency of arylsulfatase A (ASA) and the accumulation of sulfatide in several tissues. The storage material accounts for the observed metachromatic staining. MLD is a rare disease with an estimated frequency between 1:40 000 and 1:10 0000 in newborns. Arylsulfatase A catalyses the conversion of sulfatide into galactosylceramide and sulfate. For this process, the assistance of Sap-B is required. [32] MLD can be classified into a late infantile, a juvenile, and an adult form.

- Late infantile form: symptoms develop between 6 months and 4 years of age, and death usually occurs about 5 years later. It begins with hypotonia, unsteady gait, and mental regression. Several abilities progressively deteriorate. Common symptoms are loss of speech, blindness, quadriparesis, peripheral neuropathy, and seizures. In a final stage before death, the child is bedridden, in a decerebrate state, and looses all contact with his/her surroundings.
- Juvenile form: onset ranging from 4 to 16 years and death usually before 20 years.
- Adult form: can begin after puberty up to the 6th decade of life and may extend for a few years or for decades. This form is less frequent than the previous two. In both cases, patients show gradual deterioration in school or job performance, with emotional and behavioral disturbances or psychiatric symptoms in the adult form. Other clinical manifestations are gait clumsiness, incontinence, and optic atrophy. During the final stages of this disease, the patient reaches a vegetative state. [4]

The biochemical defect in all forms of MLD is a deficiency in the enzymatic hydrolysis of sulfatides. These sulfated glycolipids occur mainly in the myelin

sheaths in the white matter of the brain, in the peripheral nervous system, and in the kidney tissue. The clinical and histopathologic manifestations of MLD are fundamentally caused by a demyelination process. This phenomenon appears to be secondary to sulfatide-induced changes in oligodendrocytes and Schwann cells.

Additionally, lysosulfatide, a cytotoxic sulfatide derivative that occurs in tissues of the patients, seems to play a role in the pathogenesis of this disease.

More than 60 different mutations in the ASA gene are associated with the MLD phenotype. Some mutations produce a complete loss of enzyme activity and patients homozygous for this type of allele develop the late infantile form of the disease. On the other hand, low but definite amounts of ASA activity result in milder forms of the disease.

Also, the inherited deficiency of Sap-B, the cofactor required for sulfatide cleavage by ASA in vivo, leads to a clinical picture similar to MLD caused by ASA deficiency. In this case, the activity of ASA toward soluble, synthetic substrates is normal.

A mouse model of MLD has been created. Although it develops some neurologic symptoms, most likely due to sulfatide storage, the mice show no demyelination, which is a hallmark of the human disease. Although it shows only a low extent of neurological and neuropathological changes, this model was of value for characterization of defects in acoustic perception. To date there is no causal therapy for this lethal disease, but enzyme replacement therapy has been successfully evaluated in the animal model: In ASA knockout mice, intravenous ASA injection restored sulfatide metabolism in peripheral tissues and the central nervous system. Also, bone marrow transplantation has been applied to patients of the juvenile form. Transplantation of genetically modified stem cells gave promising results in the nervous system of the animal model.

In a related disease, multiple sulfatase deficiency known as mucosulfatidosis or Austin disease, the activities of all known sulfatases are strongly reduced. This disease results from a defective posttranslational modification, which is

necessary to enable sulfate ester hydrolysis by sulfatases. Cells from these patients show a deficient transformation of a cysteine residue into a formylglycine residue in the active site of different sulfatases. The phenotype of this disease can be described as a combination of symptoms of metachromatic leukodystrophy and a mucopolysaccharidosis. Nine mutations in the sulfatase-modifying factor-I gene (SUMFI) of seven patients have been identified that lead to deficiency of the Cα-formylglycine generating enzyme (FGE). [33] [34] [32]

## 3.6. Krabbe disease

Krabbe disease or globoid cell leukodystrophy is caused by an inherited deficiency of galactosylceramide-β-galactosidase. This membrane-associated enzyme with a molecular weight of about 50 kDa hydrolyzes galactosylceramide to ceramide and galactose. Sap-A and Sap-C are able to stimulate this degradation step in vivo. Although there is some storage especially in globoid cells, the enzyme deficiency does not lead to substantial substrate accumulation, probably because of the rapid loss of galactosylceramide synthesizing and accumulating cells. Krabbe disease and MLD are classical myelin diseases. [35]

Clinically, Krabbe disease has two variants: an infantile and a late onset form.

- Infantile form: usually start between 3 and 6 months of life, and may include irritability or hypersensitivity to external stimuli. Within a short time, severe mental and motor deteriorations occur. Commonly, patients become blind, deaf, flaccid, and hypotonic. The survival of the patients is less than two years.
- Late onset form: symptoms can appear at any time after the patients are able to walk; the onset may vary from a few years up to 73 years of age. Common clinical manifestations are psychomotor retardation, blindness, spastic paraparesis and dementia. [4]

The most characteristic histopathological changes are extensive demyelination, loss of oligodendroglia, astrogliosis, and presence of numerous multinucleated

globoid cells. These cells are hematogenous macrophages containing undigested galactosylceramide.

The pathogenesis of this disease can be attributed to a combination of two phenomena: the impaired degradation of galactosylceramide, which leads to globoid cell infiltration, and the accumulation of the cytotoxic derivative galactosylsphin-gosine (lysogalactosylceramide or psychosine), which causes oligodendroglial cell destruction. Psychosine is another substrate of the deficient enzyme; it accumulates up to toxic, cell- destructive levels. Several mutations in the galactosylceramide- β-galactosidase gene have been identified. A deletion of exons 11–17 is a very frequent mutation and is associated with the infantile form of the disease.

An authentic mouse model for Krabbe disease is the twitcher mouse with a premature stop codon within the coding sequence of the gene. Another animal model carries a mutation on the galactosylceramide-β-galactosidase gene leading to low enzyme activity. Investigations of the twitcher mouse point to a neuroinflammatory response that might additionally contribute to pathogenesis. A double knockout mouse deficient in galactosylceramide-β-galactosidase and the biosynthetic enzyme ceramide galactosyltransferase (cgt) shows shorter lifespan than the (cgt −/−) mice themselves.

A mutation within the Sap-A domain of the human and murine Sap-precursor protein causes a deficiency of the mature activator and a phenotype similar to Krabbe disease.

To date, treatment of Krabbe disease is limited to bone marrow transplantation in patients with only minimal neurologic involvement. In infantile patients, transplantation of umbilical-cord blood gave promising results, but only when the treatment was started before the onset of symptoms. Recently, peripheral enzyme replacement has been applied to Twitcher mice, which resulted in attenuation of early symptoms and an increase of life span. [35] [4]

## 3.7. Niemann–Pick disease, type A and B

Niemann–Pick disease (NPD), type A and B, is caused by the inherited deficiency of acid sphingomyelinase (ASM) and accumulation of sphingomyelin. Secondarily, sphingomyelin also accumulates in Niemann–Pick disease, type C, in which the products of the NPC-1 or NPC2 genes are deficient. In both cases, trafficking of endocytosed cholesterol is altered. Also, drug-induced lysosomal storage of sphingomyelin occurs in response to treatment of human patients with tricyclic antidepressants over long time periods. In this case. sphingomyelin accumulates due to the drug-induced degradation of acid-sphingomyelinase.

Acid sphingomyelinase is a glycoprotein with a molecular weight of 70 kDa. Within the lysosomes, it catalyses the degradation of sphingomyelin to ceramide and phosphorylcholine. Its modular structure includes a Sap-like domain and a catalytic domain. Acid sphingomyelinase can be stimulated by lysosomal lipids and sphingolipid activator proteins, but this appears not to be necessary in vivo. NPD, type A and B, is a panethnic disease with a higher frequency among Ashkenazi Jews (1:80 for heterozygotes). [36] [37]

- Type A NPD is a fatal disorder of infancy with a life expectancy of the patients of 2 to 3 years. Affected newborns appear to be normal at birth, but in the first few months of life, symptoms such as hepatosplenomegaly, moderate lymphadenopathy, hypotonia, and muscular weakness appear. Feeding difficulties and splenomegaly lead to a decrease in linear growth and body weight. In later stages of the disease, common manifestations are microcytic anemia, decreased platelet count, osteoporosis, brownish-yellow color of the skin, and cherry-red maculae. Psychomotor retardation becomes evident by 6 months of age and gradually increases over the years. In an advanced state, the patient looses contact with the environment.
- Type B NPD is a phenotypically variable disorder with little or no involvement of the nervous system. It is usually diagnosed in childhood,

but patients can reach adulthood. Commonly, liver and/or spleen are enlarged and progressive pulmonary infiltration causes the major disease complications in more severely affected patients. Patients with type B show a higher residual enzyme activity than those with type A.

NPD is characterized by the presence of “foam cells” or “Niemann–Pick cells”, although patients with other pathologies may have histologically similar cells. The formation of these histiocytic cells is caused by storage of sphingomyelin and/or other lipids in the monocyte–macrophage system. Storage occurs in spleen and lymph nodes, but also in liver, brain, kidney, and lungs. There is little or no lipid storage in the central nervous system of type B NPD patients.

Sphingolipid-induced alterations in signal transduction might also contribute to NPD pathogenesis. In response to extracellular stimuli, ceramide can be generated by hydrolysis of sphingomyelin, catalyzed by different sphingomyelinases with different topologies. Since in addition to neutral sphingomyelinases, ASM also might play a role in the activation of the so-called “sphingomyelin pathway”, NPD patients may have subtle abnormalities in various signaling pathways and these abnormalities could be exacerbated by stress.

Another factor that may contribute to the pathogenesis of the disease is the formation of sphingosylphosphocholine (SPC), a potent mitogen which can induce neurite outgrowth, and which has been shown to accumulate in type A NPD.

Different mutations in the ASM gene that cause types A and B NPD have been described. Three mutations, R496L, L302P, and fsP330, account for about 92% of the mutant alleles in Ashkenazi Jewish type A NPD patients. A common mutation in type B patients is the single lesion ΔR608.

Mouse models of NPD have been constructed by using gene-targeting strategies. Even if the precise targeting events differed in the two animal strains, they show essentially identical phenotypes. The NPD “knock-out” mice develop features of both types A and B NPD, and are used for evaluation of various

therapeutic strategies. To date, there is no specific treatment available for NPD. If performed early in life, BMT has a positive effect on the clinical course of severely affected type B NPD patients, but graft versus host disease and other transplant-related complications are common and preclude its use as a routine therapy. The outcome of BMT for the treatment of type A patients is uncertain. [4]

## 3.8. Farber disease

Farber-disease is a rare disease due to the inherited deficiency of lysosomal acid ceramidase and storage of ceramide in the lysosomes. Acid ceramidase is a heterodimeric enzyme composed of an α-subunit of 13 kDa and a β-subunit of 40 kDa. Both subunits are derived from a common 55 kDa precursor that is processed within late endosomes and lysosomes. Acid ceramidase catalyses the degradation of ceramide to sphingosine and a fatty acid in the lysosomes, and requires the presence of Sap-C or Sap-D. It is also able to catalyze the reverse reaction. [38] Point mutations on the acid ceramidase gene in patients of Farber disease have been identified; deletion of the gene leads to embryonic lethality at day 8.5 in mice.

Usually, the symptoms of Farber disease appear several months after birth, and death occurs within the first years of life.

Patients with milder forms of the disease can reach adulthood. The most characteristic clinical manifestation is the development of painful and progressive joint deformations, subcutaneous nodules (lipogranulomas), and progressive hoarseness. In the granulomas, but also in some organs and tissues, lipid-leaden macrophages are frequent. Apart of skin and joints, liver, spleen, lung, and heart are frequently affected organs. Neuronal accumulation of ceramide and gangliosides has also been reported. A variant form of the disease is present in prosaposin (Sap-precursor) deficiency, where the resulting deficiency also of Sap-D prevents ceramide degradation. The biochemical

findings show combined characteristics of Farber disease, and at least four other sphingolipidoses.

The important role of ceramide in skin function accounts for the involvement of subcutaneous tissues in Farber disease. The accumulated lysosomal ceramide in Farber disease does apparently not cause apoptosis or another response expected for this signaling substance. The clinical course of this disease correlates with the residual acid ceramidase activity. Therapy of infantile ceramidase deficiency has been attempted with bone marrow transplantation, which improves the peripheral, but not the neurological manifestations. [4]

## 3.9. Deficiency of Saposins (Saps)

- **Prosaposin:** All four saposins are derived from a single protein, the Sap-precursor, or prosaposin, a 70 kDa glycoprotein, which is proteolytically processed to the mature activator proteins (Saps) in the late endosomes and lysosomes. Prosaposin is detected mainly in brain, heart, muscle and body fluids, whereas mature Saps are mainly found in liver, lung, kidney and spleen. Until now, two different mutations in few human patients have been reported, a homoallelic mutation of the start codon and a homoallelic deletion within the Sap-B domain, which leads to a frame-shift and a premature stop codon. The Sap-precursor deficiency is a fatal infantile storage disorder, characterized by hepatosplenomegaly and severe neurological symptoms. In all human patients, but also in the Sap-precursor knockout mouse, there is simultaneous storage of many sphingolipids, including ceramide, glucosylceramide, lactosylceramide, ganglioside GM3, galactosylceramide, sulfatides, digalactosylceramide, and globotriaosylceramide, accompanied by an accumulation of intralysosomal membranes. Prosaposin deficiency can be diagnosed by immunochemical methods, by demonstration of a metabolic defect, or with antisera against more than one Sap protein. [39]

- **Sap-A:** required for the degradation of galactosylceramide by galactosylceramide-β-galactosidase in vivo. Mice carrying a mutation in the Sap-A domain of the Sap-precursor protein show accumulation of galactosylceramide and the late-onset form of Krabbe disease. A human disease has been described that resembles Krabbe disease, but is caused by a singular defect of Sap-A. [40]
- **Sap-B deficiency:** is the first sphingolipid activator protein that was identified. It is a lipid-transport protein which shows a broader specificity than the GM2-activator. It is able to stimulate the degradation of 20 glycolipids in the presence of human, plant, and bacterial enzymes. In vivo, it mediates the degradation of sulfatide by arylsulfatase A, of globotriaosylceramide and digalactosylceramide as demonstrated in patients with Sap-B deficiency, where these substrates accumulate in the urine. It is also required for the degradation of ganglioside GM3 and lactosylceramide, as reported by studies in cultured human skin fibroblast of Sap-B deficient patients. The crystal structure of unglycosylated human recombinant Sap-B has been solved. The inherited defect of Sap-B leads to an atypical form of metachromatic leukodystrophy, with late infantile or juvenile onset. The disease is characterized by accumulation of sulfatides, digalactosylceramide, and globotriaosylceramide. The clinical findings in Sap-B deficiency are similar to those in metachromatic leukodystrophy. Nine cases with early juvenile and juvenile onset have been reported. [41]
- **Sap-C deficiency**: was first isolated from spleens of patients of Gaucher disease. It is required for the lysosomal degradation of glucosylceramide by glucosylceramide-β-glucosidase and lends protease-resistance to the enzyme inside the cell. Recently, the solution structure of Sap-C has been solved. In contrast to the mode of action of the GM2-activator and of Sap-B, Sap-C can activate glucosylceramide-β-glucosidase directly. Sap-C deficiency leads to an unusual juvenile form of Gaucher disease and an

accumulation of glucosylceramide mainly in brain, liver and spleen. Until now, only two human patients with point mutations in one allele of the Sap-C domain of prosaposin have been diagnosed. The mutations led to the substitution of the same cysteine residue and the subsequent loss of a disulfide bridge in the variant proteins. The clinical findings are similar to those in Gaucher disease, type 3. Until recently, the other allele remained unidentified in the two patients. A recent work demonstrated a Q430X mutation in the Sap-D-domain on the other allele, so that at least one of the patients is compound heterozygote for Sap-C- and prosaposin deficiency. [42]

- **Sap-D:** stimulates lysosomal ceramide degradation by acid ceramidase in in-vitro and in cultured cells. In Sap-D deficient mice, ceramides, particularly those containing hydroxy fatty acids, accumulate in the kidney and the brain of the animals, which, however, suffer also from severe urinary system defects. Human diseases based on the isolated defect of this cofactor are unknown to date. [43]

# 4. Animal models of Gangliosidosis

Naturally occurring animal models of GM2-gangliosidoses have been reported in several species. Human classification of Gangliosidoses has been used even for animal diseases. In addition, murine models of Tay–Sachs disease, Sandhoff disease and GM2-activator deficiency have been generated using gene-targeting techniques [98]. [44]

## 4.1. Murine (induced)

- **GM1**: β-Gal null mice accumulate GM1 ganglioside and its asialo derivative, GA1, in the CNS prior to symptoms, which are tremor, ataxia and gait abnormalities. The lifespan of the mice is 7–10 months. In general, this mouse model faithfully recapitulates the human disease, specifically the infantile form, although they do not display hepatosplenomegaly and skeletal dysplasia. The efficacy of substrate reduction therapy has been investigated in these mice, as well as gene therapy. This mouse has also proven useful in obtaining mechanistic insight into the GM1 gangliosidosis; thus, GM1 accumulates in the endoplasmatic reticulum causing changes in calcium flux and in the un-folded protein response. A transgenic mouse of the milder juvenile form was created by introducing the R201C mutation. Chemical chaperone therapy has been tested on this mode [44]
- **GM2**: Several mouse models have been generated. Hexa−/− mice suffer no obvious behavioral or neurological deficits while Hexb−/− mice develop a fatal neurodegenerative disease. Interestingly, Hexa−/− female mice with repeat breeding histories develop severe progressive hind limb weakness with impaired motor coordination, balance, and mild ataxia; thus, even though the Hexa−/− mouse is asymptomatic, it can be used as a model of pregnancy-induced late onset Tay–Sachs disease. Mice that have both Hexa and Hexb genes disrupted display the phenotypic,

pathologic and biochemical features of gangliosidosis. Mouse models for GM2 activator deficiency express an intermediate phenotype between Tay–Sachs and Sandhoff. Two inducible strains, Hexb−/− HexTg and Hexb −/− SYNTg, have been generated, in which DOX is used to regulate Hexb levels; near-total gene silencing is achieved upon DOX-treatment which can be reversed by DOX withdrawal. [44]

Some phenotypic differences between mice and humans may result from differences in ganglioside degradation between the two species: in the mouse model of Tay–Sachs disease, sialidases are able to convert GM2 to GA2, which is then degraded through a minimal yet sufficient activity of HexB and GA2 degradation by HexB results from a mouse GM2 activator protein that is much less restrictive than its human counterpar. Thus, an alternative catabolic pathway for GM2 ganglioside in mice is responsible for a Tay–Sachs model that does not faithfully recapitulate the human condition. [25]

## 4.2. Swine

In 1976 has been described GM2 gangliosidosis in Yorkshire pigs. Autosomal recessive mode of inheritance was confirmed.

Clinically affected pigs (homozygous) were normal size at birth but have a reduced post-natal growth rate than their normal or heterozygous (carrier) littermates. Incoordination, evident at 3 months old, manifested by an abnormal gait (“goose-stepping”) involving both rear legs. The incoordination become increasingly severe until they become recumbent at 4-6 month of age. Numerous tiny white spot diffusely scattered through the retina have been seen. Prominent dark blue cytoplasmic granules were present in neutrophils with an increase frequency of azurophilic cytoplasmic granules in lymphocytes.

Histologically neurons throughout the brain and spinal cord were characterized by foamy or diffusely vacuolated cytoplasm, that stained with toluidine blu; ganglion cells of the myoenteric plexus appeared to be affected, such as microglial cells, reticuloendothelial cells and neutrophils. Electron microscopy

(TEM) revealed that cytoplasmic inclusions were membrane-bounded, 0.6-1.0 μm in diameter and partially or completely filled with membranes arrayed in a laminar pattern.

Biochemically, there was less β-Hexaminidase in tissue homogenates from affected than normal pigs, but there was more in serum of affected than carrier or normal ones; Hexaminidase A isoenzyme was severely reduced in affected pigs and Hexaminidase B approximates that of the normal ones. Cerebral cortical ganglioside was 2-4 time higher than normal; $GM_2$ ganglioside was the 58% of total gangliosides in affected pigs.

These $GM_2$ gangliosidosis has many features in common with AB variant in human pathology (later age of onset and death, no cherry red spot in the retina, no macrocephaly, late onset of blindness, moderate increase in total ganglioside content of the brain and partial deficiency of isoenzyme A Hexaminidase); particularly, low levels of Hexaminidase activity, especially the isoenzyme A with a normal B, are similar to the AB variant. Although the morphology of the cytoplasmic inclusion and the degree of neuronal involvement are similar to human Tay-Sachs and Sandhoff diseases.

No data are available on genetic and amminoacidic defects in these animals. [45] [46]

## 4.3. Canine

The first documented ganglioside storage disease of domestic animal was a case of GM2 Gangliosidoses in German Shorthair Pointer dogs in 1967. [47]
GM1 has been diagnosed in several breeds:

- **Beagle**: A 9-month-old dog with a history of progressive motor dysfunction was shown to have a deficiency in brain beta-galactosidase activity. The canine disease, like that of children with GM1 gangliosidosis, is characterized by accumulation of GM1 ganglioside in the brain, liver, and spleen, and membranous cytoplasmic bodies in neurons [48]
- **English springer spaniel**: two litters of English springer spaniel puppies showed a progressive neurological impairment, dwarfism, orbital hypertelorism, and dysostosis multiplex. An excess of GM1-ganglioside was found in the brain. Three abnormal oligosaccharides were present in samples of urine, brain, liver, and cartilage [49]
- **Portuguese water dogs**: Three Portuguese water dog siblings, all females aged 5 to 7 months, were killed following a brief period of neurologic disease. GM1 ganglioside in brain was markedly elevated in all three dogs, and beta-galactosidase activity was less than 10% of control values. [50]
- **Shiba**: A six-month-old shiba dog with a one-month history of progressive motor dysfunction showed clinical signs of a cerebellar disorder, including ataxia, dysmetria and intention tremor of the head. The activities of lysosomal acid beta-galactosidase in its leucocytes and liver were less than 2 per cent of the control levels, and the compound accumulated in the brain was identified as GM1 ganglioside. A sibling which died immediately after birth was shown to have a beta-galactosidase deficiency in the brain and visceral organs. A family study revealed that the sire and dam of the probands were heterozygotes with approximately half of the normal level of beta-galactosidase activity. [51]

- **Cross-bred**: 13-month-old dog with 3-month history of progressive ataxia, paraparesis, intention tremor and regurgitation. On neurological examination, the dog was found to have a depressed mental status and a variety of signs indicative of brain stem and cerebellar dysfunction. [52]
- **Alaskan Huskies**: two females and one male, were diagnosed with GM1-gangliosidosis. Clinically, diseased animals exhibited proportional dwarfism and developed progressive neurologic impairment with signs of cerebellar dysfunction at the age of 5–7 months. Skeletal lesions characterized by retarded enchondral ossification of vertebral epiphyses were revealed by radiographs of the male dog at 5.5 months of age. [53]

*Tab. 3 Canine breeds, their genetic defect in GLB1 gene and amino acid change in GM1 gangliosidosis*

| Genetic defect in GM1 (gene GLB1) | | | | |
|---|---|---|---|---|
| **Breed** | **Genetic defect** | **Exon** | **Amino acid change** | **Ref** |
| **Portuguese water dog** | 200g>a | 2 | R60H | [54] |
| **Alaskan husky** | 1688_1706dup19 | 15 | rame shift, PTC and exon 15 skipping | [55] |
| **Shiba dog** | 1668delC | 15 | Frame shift and PTC | [54] |

GM2 has been diagnosed in several breeds:

- **German shorthair pointer** [56]
- **Golden retriever**: A golden retriever dog with total hexosaminidase deficiency and raised GM2-ganglioside in CSF [57]
- **Toy poodle:** Three red-haired Toy Poodles demonstrated clinical signs including motor disorders and tremor starting between 9 and 12 months of age. The animals finally died of neurological deterioration between 18 and 23 months of age. Biochemically, GM2 ganglioside had accumulated in the brain, and Hex A and Hex B were deficient in the brain and liver.

Pedigree analysis demonstrated that the 3 affected dogs were from the same family line [58]

- **Japanese chin**: in a 2-year-old massive storage of GM2 ganglioside as well as a seemingly paradoxical increase in total beta-hexosaminidase activity measured in vitro. [59]. A15-month-old, male neutered Japanese Chin dog was presented for evaluation of blindness and an abnormal gait first observed at 11 months of age. On presentation, the dog was periodically obtunded and had cerebellar ataxia with spastic, hypermetric postural reactions. The dog had an absent menace response and ventro-lateral strabismus in both eyes. A 18-month-old, male neutered, Japanese Chin dog was presented for evaluation of progressive mental dullness of 2 months duration. On neurologic examination, the dog was ambulatory with moderate cerebellar ataxia and intention tremors. Menace response was absent OU. [60]
- **Mix-breed:** simultaneous deficiencies of Hex A and Hex B; Immunohistochemical analysis demonstrated the accumulation of GM2 ganglioside in the cytoplasm of neurons and tested negative for the mutation of the canine *HEXB* gene that causes SD in toy poodles [61]

*Tab. 4 Canine breeds, their genetic defects and amino acid change in GM2 gangliosidosis*

| Genetic defect in GM2 | | | | | |
|---|---|---|---|---|---|
| **Breed** | **Gene** | **Genetic defect** | **Exon** | **Amino acid change** | **Ref** |
| **Japanese Chin** | HEX A | c.967G>A | 8 | Sobstitution p.E323K | [62] |
| **Toy Poodle** | HEX B | C.283delG | 3 | p.V59fsX | [63] |

## 4.4. Feline

GM1 gangliosidosis

- **Siamese**: affected kitten have little storage in affected organs, similar to the juvenile onset (type II) of human disease [64]
- **Domestic short-hair**: several families have been identified [65] [66] [67] [68]
- **Korat:** A 7-month-old Korat cat was referred for a slowly progressive neurological disease. Circulating monocytes and lymphocytes showed the presence of single or multiple empty vacuoles and blood leukocytes enzyme assay revealed a very low beta-galactosidase activity level (4.7 nmol/mg per h) as compared to unaffected parents and relatives [69]

*Tab. 5 Feline breeds, their genetic defect in GLB1 gene and amino acid change in GM1 gangliosidosis*

| Genetic defect in GM1 | | | | | |
|---|---|---|---|---|---|
| **Breed** | **Gene** | **Genetic defect** | **Exon** | **Amino acid change** | **Ref** |
| **Siamese, Korat, Asian domestic short-hair** | GLB1 | c.1448G>C | 16 | Substitution analogous to Arg482His/Cys | [70] |
| **Japanese domestic** | GLB1 | No c.1448G>C | - | - | [68] |

GM2 gangliosidosis

- **Domestic short-hair**: several families have been identified [71] [72] [73]
- **Korat:** marked deficiency in the activity of hexosaminidase (HEX) A and B in affected brain and liver as compared to controls. Electrophoresis of a liver extract revealed a deficiency of normal HEX A and B in the affected animals. Animal model of human Sandhoff's disease. [74]

- **Eurpean Burmese**: deficient activity of both HexA and HexB accompanied by elevated levels of other lysosomal enzymes such as b-galactosidase and a-mannosidase [75]

*Tab. 6 Feline breeds their genetic defects and amino acid change in GM2 gangliosidosis*

| Genetic defect GM1 | | | | | |
|---|---|---|---|---|---|
| **Breed** | **Gene** | **Genetic defect** | **Exon** | **Amino acid change** | **Ref** |
| **Domestic shorthair** | GM2 A | c.516_519 delGGTC | terminal exon | omits 1 amino acid of the GM2 activator protein, alters the reading frame for the next 15 amino acids, and introduces a premature termination codon that is 5 amino acids premature | [76] |
| **Korat** | HEX B | c.39delC | - | frame shift and introduction of a stop codon at position 191 | [77] |
| **Domestic shorthair** | HEX B | c.1467_14 91linv25 | 14 | introduces three amino acid substitutions at the carboxyl terminus of the protein and a translational stop that is eight amino acids premature | [78] |
| **Japanese domestic Cat** | HEX B | c.667C>T | 7 | codon of arginine was altered to a stop codon by the mutation (R223X), resulting in truncation of *HEXB* products in which a 277-amino-acid sequence at the C terminal region was deleted. The other clone lacked the entire exon 7 sequence of the *HEXB* gene | [79] |

| | | | | | |
|---|---|---|---|---|---|
| **Burmese** | HEXB | c.1244-8_1250del115 | 12 | removal of intron 11, exon 12, and intron 12. Splicing of exon 11 directly to exon 13 caused an 18 amino acid substitution followed by premature termination at codon 472, compared to the normal protein of 538 amino acids | [75] |

## 4.5. Other species

- **Bovine**: GM1 is the only gangliosidosis that has been described in Bovine; Fresian calves are the affected breed, characterized by Beta-galactosidase deficiency [80]
- **Sheep**: in Suffolk sheepes has been described GM1 gangliosidosis with onset of neurologic signs at 4–6 months; biochemical and enzymatic evaluation disclosed storage of GM1 ganglioside and neutral long chain oligosaccharides in brain, urinary excretion of neutral long chain oligosaccharides, and deficiencies of lysosomal β-galactosidase and α-neuraminidase. [81] In Coopworth Romney-cross sheep has been described GM1 gangliosidosis with deficiency (90%) of acidic beta-D-galactosidase. [82] In Romney sheep has been described GM1 gangliosidosis similar to human type 3 [83] In American Jacob sheep has been described GM2 gangliosidosis with ataxia in all 4 limbs, proprioceptive deficits, and cortical blindness; hexosaminidase activity was reduced in the affected sheep to 29%; mutation was demonstrated at nucleotide position 1330 (C1330→C), which resulted in an amino acid change from Gly444 to Arg. [84] In the English Jacob Sheep, serum biochemistry detected a marked decrease in hexosaminidase A activity in the one lamb tested, when compared with the concentration in age

matched controls and genetic analysis identified a mutation in the sheep hexa allele G444R (as the american). [85]

- **Flamingo**: with Hex A deficiency occurring spontaneously in nature; accumulation of GM2-ganglioside and a homozygous P469L mutation in exon 12 of the HEXA gene. [86]
- **Deer**: two juvenile siblings male Muntjak deer (*Muntiacus muntjak*) with histories of depression, ataxia, circling and visual deficits. deficiency of β-hexosaminidase A. [87]
- **Rabbit**: A 1.5-year-old neutered male rabbit was presented with chronic nasal discharge and ataxia; markedly reduced activity of tissue b-hexosaminidase A complete absence of the enzymatic activity of the a-subunit of that enzyme GM2 gangliosidosis variant B1 [88]
- **Black bear**: ages 10-14 months in poor clinical condition, lethargic, tremulous and ataxic. Acid β-galactosidase activity in cultured skin fibroblasts was only 1-2% of control values. homozygous recessive T(1042) to C transition inducing a Tyr348 to His mutation (Y348H) within a highly conserved region of the GLB1 gene. [89]
- **Emu**: two, 7 e 6-month-old female, emu (Dromaius novaehollandiae) died following acute central nervous system signs. 14- and 25-fold increases of GM1 and GM3 gangliosides. [90]

## 4.6. Disease models for the development of therapies

Highly predictive *in vitro* and *in vivo* preclinical disease models are especially critical for rare diseases, including LSDs, where the pediatric age range of the disease populations and small number of patients make the design of clinical studies very challenging, particularly to standardize endpoint measurements and measure statistically significant differences between the drug-treated and control group. [3]

Predictive *in vitro* cell-based assays with human disease cells, for example, cells derived from patient induced pluripotent stem cells (iPSCs), can provide critical efficacy data to help guide clinical development. Patient iPSC-derived cells are being produced as alternative preclinical models to aid in understanding the pathological pathways causing LSDs and to serve as predictive assay platforms for drug development. These cell-based disease models facilitate the identification of new targets and biomarkers and the screening of small-molecule libraries for drug discovery.

Proof-of-concept studies of candidate therapeutic molecules for LSDs require rigorous testing in animal models with molecular pathophysiology and clinical phenotype as close to the human disease as possible in order to improve the predictability of drug efficacy in human clinical trials. [3]

Genetically engineered mouse models are frequently used for *in vivo* preclinical efficacy studies, and many knockout or knockin models for LSDs have been reported. However, LSD mouse models have not always been successful in accurately reproducing the human disease phenotype [3]. Particularly, as said before, an alternative catabolic pathway for GM2 ganglioside in mice is responsible for a Tay–Sachs model that does not faithfully recapitulate the human condition. [25] Even though, mouse models are still valuable and will continue to be used for initial studies because they are inexpensive, available in well-characterized inbred strains, and easy for introducing genetic modifications and producing large colonies of animals in a short time in order to test the effects

of an adequate number of compounds *in vivo* for further selection of lead therapeutic development candidates. [3]

For *in vivo* testing of potential therapeutic candidates, large animal models, with spontaneous natural, hold promise as models for improving the predictability of human disease phenotypes and efficacy. These animal models have supported LSD drug approvals by contributing to a better understanding of the natural history of disease progression, defining a drug candidate's pharmacokinetic and pharmacodynamic relationship and defining clinical endpoints. Furthermore, they offer several advantages in the translatability of preclinical studies to clinical trials that can complement and provide additional value to studies in mice. Large animal models for disease in which the size and complexity of the brain are more similar to humans have allowed a comparison of different CNS delivery methods for safety, distribution, and efficacy of potential therapeutics; these species also have more heterogeneity in genetic backgrounds, respecting to mouse models, which better mimics human populations. [3] [25] [91]

# 5. Laboratory diagnosis of Gangliosidosis

## 5.1. Histology and Transmission Electron Microscopy

On histologic examination, the neurons are ballooned with cytoplasmic vacuoles, each representing a markedly distended lysosome filled with gangliosides; this process occurs in brain, cerebellum as well as in neurons throughout the basal ganglia, brain stem, spinal cord, and dorsal root ganglia and in the neurons of the autonomic nervous system. The ganglion cells in the retina are similarly swollen particularly at the margins of the macula. Different stains are positive: Periodic acid–Schiff stain (PAS) in frozen tissue, oil red O, Sudan black B, Toludine blue and Luxol fast blue. [1] Vacuolated macrophages may also be found around blood vessels in the CNS, and storage also occurs in glial cells. Axonal spheroids may be reasonably numerous. Golgi studies reveals spout ectopic dendritic processes that received normal-appearing synaptic inputs (ectopic dendritogenesis). [12] Gliosis, demyelination, and neuronal loss are not apparent until end stage. In those instances, where hepatic storage occurs, hepatocytes and Kupffer cells contain large, empty vacuoles, the site of storage of a water-soluble glycopeptide. [92]

In transmission electron microscopy, specific ultrastructural aspects of the lysosomal inclusions are characteristic for certain syndromes: tubules in Gaucher's and Krabbe's diseases, prismatic structures in metachromatic leukodystrophy, clear vacuolar inclusions in the mucopolysaccharidoses, lamellar structures of gangliosidosis and the Niemann-Pick and Fabry's syndromes, and the curvilinear membranes of Farber's disease. [93] Gangliosidosis are characterized by numerous variably in sized cytoplasmic inclusions; the most prominent are whorled configurations within lysosomes composed of onion-skin layers of membranes (cytoplasmic membranous bodies). In time, there is progressive degeneration and loss of neurons,

proliferation of microglia, and accumulation of complex lipids in phagocytes within the brain substance. [1] [94] [95]

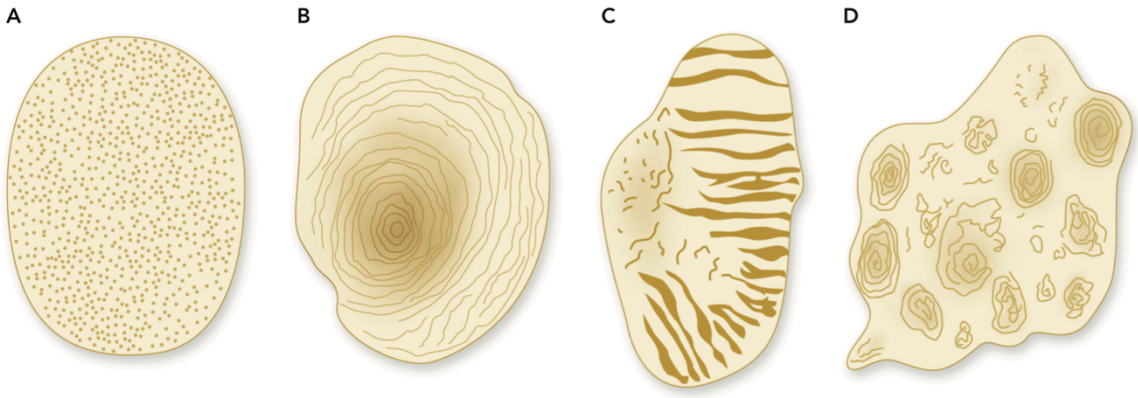



*Fig. 11 Schematic morphology of storage compartments commonly observed in lysosomal storage disorders cells. A: floccular-granular storage. B: lipid whorls. C: zebra bodies. D: autophagic vacuoles [96]*

## 5.2. Biochemical analysis

Assaying for the activities of a selection of lysosomal enzymes, provides a definitive diagnosis for the majority of lysosomal storage diseases. An age-matched control should be assayed in parallel to provide a normal set of control values. For autosomal recessive diseases, an affected homozygote would be expected to have severely depleted enzyme activity (typically 0–5% of normal), whereas heterozygotes should have approximately 50% of the normal activity. Thus, heterozygotes may be diagnosed through enzyme assay, particularly when pedigree information can assist in the interpretation of results. Even if the precise enzyme is not identified in a preliminary screen, the secondary increase observed in the activities of other lysosomal enzymes when one is deficient is enough to merit further investigation. As the level of conservation of lysosomal enzyme structure between species is high, enzyme activities in animals are investigated by the same techniques and artificial substrates as those used in their human counterparts. [97]

For biochemical study of gangliosidosis, the assay of β-hexosaminidase and β-galactosidase or thin layer chromatography for GM1 and GM2 are the standard evaluations:

- **Assay of β-hexosaminidase and β-galactosidase**: The protein content of leukocyte samples is extracted and determined. The substrate for leukocytes enzyme activity are 1.5 mM 4-methylumbrelliferyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside for β-hexosaminidase, 1 mM 4-methylumbelliferyl-β-galactopyranoside for β-galactosidase. Substrate hydrolysis is measured spectrofluorometrically at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Specific activities were reported as nmol substrate cleaved/mg protein per hour. Activities are variably reduced, depending on the alteration of the enzyme. [98] [69]
- **Analysis of β-hexosaminidase by electrophoresis**: Electrophoresis of both plasma and leukocyte lysates carried out on cellulose acetate membranes; the membrane was incubated between two filter papers impregnated with substrate solution (2 mM 4-MUG or 4-MUGS in 0.1 M citrate–phosphate buffer, pH 4.4). After incubation, the membrane was developed with concentrated ammonium hydroxide and illuminated with ultraviolet light (312 nm). The bands of fluorescent-free 4-MU representing Hex activity; in Sandhoff disease there are no bands. [98] [69]
- **Analysis of GM1 and GM2 ganglioside with Thin-layer chromatography (TLC)/enzyme-immunostaining**: High performance thin-layer chromatography (HPTLC) performed on 20 × 20 cm silica gel HPTLC. The mobile phase was chloroform/methanol/0.2% calcium chloride (50:42:11) for Lower Phase and chloroform/methanol/ammonia (80:20:2) for Upper Phase. TLC of LP was developed by the resorcinol sulfuric acid reaction, while TLC of UP was stained with 0.3% ninhydrin in a butyl alcohol and 0.03% acetic acid solution. A densitometric analysis carried out on HPTLC stained bands. The relative absorption of the GM1 and GM2 ganglioside bands to the total stained ganglioside bands are expressed as a percentage. [98] [69]

## 5.3. Genetical analysis

Lysosomal storage diseases are rewarding subjects to genetical study because they are single gene defects and are inherited in an autosomal recessive pattern. [97]

Numerous mutations, in patients affected by gangliosidosis, are well characterized in human and veterinary medicine. This allowed the development of a real-time PCR genotyping assays for rapid evaluation of a single patient disease or large-scale genotyping and screening for reported mutation. Particularly in veterinary medicine, genotyping surveys are carried out in different reported species, to determine the current mutant allele frequency. [99] New forms of the same disease may be recognized, however, and the experience in human medicine is that almost each affected family has a novel mutation; therefore, excluding the reported mutation in an unrelated case with suspicious clinical signs cannot categorically rule out the presence of a specific disease. [97] In these cases, broad spectrum next generation sequencing (NGS) genetic testing using either gene panels, whole exome sequencing (WES) (which queries the entire coding sequence of the genome), or whole genome sequencing (WGS) are recommended. [100]

# 6. Gangliosidosis in a family of wild boars

## 6.1. Clinical presentation

The subjects of this study came from a partial free-range farm in Emilia Romagna (Italy). The farm was composed by 2 females and 1 male boar. In April 2014, a litter of 3 boars was born, 2 males and 1 female. At 6 months of age one male presented neurological sign: behavioral changes, dysmetria and ataxia. Viral and bacterial analysis were performed and resulted negative for bacterial culture, Classical Swine Fever (*Flaviviridae, Pestivirus*) and Aujeszky (*Herpesvirus, Alphavirus*). Plant's and drugs toxicosis were excluded. [101] Different samples of blood, serum and plasma were takem; PBMC One month later the other littermates began to present the same neurological signs. At 9 months, the first affected subject presented involuntary twitching, seizures, decrease in body condition, quadriplegia and lateral decubitus; due to the worsening conditions the subject was euthanized with an intramuscular injection of sedative (Stresnil® 0,50ml / 20Kg) followed by an intravenous injection of sodium Thiopental, used in overdose (200mg / Kg). Necropsy was performed by Istituto Zooprofilattico of Lombardy and Emilia Romagna. Histological samples have been sent to Operative unit of Veterinary Pathology of University of Parma. The remnants 2 boars worsening their neurological symptoms, characterized by quadriplegia and lateral decubitus, head and limb tremors. Due to the worsening conditions the subjects were euthanized at 1 year of age with an intramuscular injection of sedative (Stresnil® 0,50ml / 20Kg) followed by an intravenous injection of sodium Thiopental, used in overdose (200mg / Kg). Necropsy was performed by Operative unit of veterinary Pathology of University of Parma.

## 6.2. Methods

### 6.2.1. Histology

Samples were taken during necropsy examination. Different samples of lung, heart, thymus, liver, pancreas, spleen, gastrointestinal tract, lymph nodes, kidney, adrenal, skeletal muscle, skin, eye, spinal cord, brain and pituitary gland were taken. Brain sections collected are:

- Frontal lobes
- Basal ganglia
- Caudate nucleus
- Thalamus
- Hippocampus
- Cerebellum
- Pons

Same samples were collected from two age- and sex-matched normal wild board for comparison.

Tissues were fixed in 10% of neutral buffered formalin; they were trimmed and passed through a series of alcohol-water solutions, beginning with 75 percent up to absolute alcohol, xylene and heated paraffin (Automated tissue processing, Bio-Optica, VTP300**)**. They were finally embedded on paraffin blocks. Cut sections at a thickness of about 4-5 μm were obtained with microtome (Leica); microscope slides with cut sections were later dried overnight at 37° C.

Sections were stained with Hematoxylin and Eosin (H&E):

1. Deparaffinize sections on xylene;
2. Hydration: Hydrate the tissue section by passing through decreasing concentration of alcohol (100%, 90%, 70%) up to water;
3. Stain in Hematoxylin for 5 minutes;
4. Wash in running tap water for 5 minutes;
5. Stain in 1% Eosin for 1 minutes;

6. Wash in tap water for 5 minutes;
7. Dehydrate in increasing concentration of alcohols and clear in xylene;
8. Mount in mounting media. [102]

Histological slides were examined with Nikon Eclipse E800 microscope (Nikon Corporation, Japan) using Nikon PLAN APO lenses. Sections were photographed with Camera DIGITAL SIGHT DS-Fi1 (Nikon Corporation, made in Japan); pictures were acquired with DS Camera Control Unit DS-L2 (Nikon Corporation, Japan) and stored in USB device.

### 6.2.2. Histochemical stainings

- **Periodic acid-Schiff (PAS, Bio-Optica 05-M05030)**:
    1. Deparaffinize and hydrate to water;
    2. Oxidize in 0.5% periodic acid solution for 5 minutes;
    3. Rinse in distilled water;
    4. Place in Schiff reagent for 15 minutes (Sections become light pink color during this step);
    5. Wash in lukewarm tap water for 5 minutes (Immediately sections turn dark pink color);
    6. Counterstain in Mayer's hematoxylin for 1 minute;
    7. Wash in tap water for 5 minutes;
    8. Dehydrate and coverslip using a mounting medium. [103]

- **Toluidine blue (resin-embedded sections):**
    1. Cut thick sections at 0.5 um or 1.0 um;
    2. Use a metal loop to collect thick sections, and transfer sections to a drop of distilled water on a glass slide;
    3. Dry sections down on a glass slide by placing the slide on a slide warmer or 40 wt lamp;

4. After the sections are completely dried, cover with a few drops of staining solution (Toluidine blue 0.5% sodium carbonate) for 1-2 minutes, depending on the darkness of staining to achieve;
5. Rinse off excess stain gently with distilled water;
6. Air dry the slide;
7. Coverslip with regular mounting medium. [104] [105]

- **Luxol Fast blue (Bio-Optica, 04-2008120):**
  1. Deparaffinize and hydrate to 95% ethyl alcohol;
  2. Leave in luxol fast blue solution in 56° C oven overnight;
  3. Rinse off excess stain with 95% ethyl alcohol;
  4. Rinse in distilled water;
  5. Differentiate the slides in the lithium carbonate solution for 30 seconds;
  6. Continue differentiation in the 70% ethyl alcohol for 30 seconds;
  7. Rinse in distilled water;
  8. Check microscopically to see if gray matter is clear and white matter sharply defined;
  9. Repeat the differentiation steps (step 5-7) if necessary;
  10. When differentiation is complete, place in distilled water;
  11. Counterstain in the cresyl violet solution for 30-40 seconds;
  12. Rinse in distilled water;
  13. Differentiate the slides in 95% ethyl alcohol for 5 minutes (check microscopically);
  14. 100% alcohol 5 min;
  15. 100% alcohol 5 min;
  16. Xylene 5 min;
  17. Xylene 5 min;
  18. Coverslip with regular mounting medium. [106]

### 6.2.3. Immunohistochemical stainings

- **GFAP (Glial fibrillary acidic protein, G-3893, Sigma-Aldrich):**
    1. Deparaffinize and hydrate to water;
    2. Antigen Retrieval: immersing the sections in citrate buffer and perform antigen retrieval using microwave (3minutes X3, 400W);
    3. Cool section at room temperature for 20 minutes;
    4. Peroxidase Blocking Solution (3% $H_2O_2$ in PBS) for 12 minutes;
    5. Primary Antibody: incubate sections in GFAP primary antibody (Sigma-Aldrich) at dilution 1:400 in bovine serum albumin overnight at 4°;
    6. Rinse in PBS;
    7. Secondary Antibody: incubate sections in Biotinylated secondary anti-mouse antibody (Vector Laboratories) in PBS for 30 minutes at room temperature;
    1. Rinse in PBS;
    2. Detection: incubate sections in ABC-Peroxidase Solution (Vectastain) for 30 minutes at room temperature;
    3. Rinse in PBS;
    4. Chromogen/Substrate (DAB): incubate sections in peroxidase substrate solution 2 minutes;
    5. Rinse in PBS;
    6. Counterstain with Hematoxylin solution;
    7. Rinse in running tap water for 2-5 minutes;
    8. Dehydrate through 95% ethanol for 1 minute, 100% ethanol for 2x3min;
    9. Clear in xylene for 5minutes;
    10. Coverslip with mounting medium [107].

- **Vimentin (Clone V9, Dako):**

1. Deparaffinize and hydrate to water;
2. Antigen Retrieval: immersing the sections in citrate buffer perform antigen retrieval using microwave (3minutes X3, 400W);
3. Cool section at room temperature for 20 minutes;
4. Peroxidase Blocking Solution (3% H2O2 in PBS) for 12 minutes;
5. Primary Antibody: incubate sections in Vimentin primary antibody (Dako) at dilution 1:50 in bovine serum albumin for 1 hour at room temperature;
6. Rinse in PBS;
7. Secondary Antibody: incubate sections in Biotinylated secondary anti-mouse antibody (Vector Laboratories) in PBS for 30 minutes at room temperature;
8. Rinse in PBS;
9. Detection: incubate sections in ABC-Peroxidase Solution (Vectastain) for 30 minutes at room temperature;
10. Rinse in PBS;
11. Chromogen/Substrate (DAB): incubate sections in peroxidase substrate solution;
12. Rinse in PBS;
13. Counterstain with Hematoxylin solution;
14. Rinse in running tap water for 2-5 minutes;
15. Dehydrate through 95% ethanol for 1 minute, 100% ethanol for 2x3min;
16. Clear in xylene for 5 minutes;
17. Coverslip with mounting medium. [107]

*Tab. 7 Used antibodies, sources, pretreatments, dilutions, secondary antibodies and revelation method for Immunohistochemistry*

| Immunohistochemistry | | |
|---|---|---|
| | **GFAP** | **VIMENTIN** |
| **Antigen Retrieval** | 3x3' 400 Watt MW | 3x3' 400 Watt MW |
| **Buffer** | citrate buffer pH 6±0.2 | citrate buffer pH 6±0.2 |
| **Peroxidase Blocking Solution** | 3% H2O2 in distilled water 12' | 3% H2O2 in distilled water 12' |
| **Primary Antibody** | Monoclonal mouse Anti-Glial Fibrillary Acidic Protein (GFAP) Clone G-A-5 Sigma-Aldrich | Monoclonal Mouse Anti-Vimentin Clone V9 Dako |
| **Incubation** | Overnight, 4° C | 1 h, room temperature |
| **Secondary Antibody** | Biotinylated Goat anti-mouse IgG | Biotinylated Goat anti-mouse IgG |
| **Detection System** | ABC-Peroxidase Solution (Vectastain) | ABC-Peroxidase Solution (Vectastain) |

### 6.2.4. Transmission Electron Microscopy (TEM)

Tissue were fixed in 2.5% glutaraldehyde / 0.1M PBS buffer pH 7.2 for 1 hours and then dehydrated through the graded series of acetone and embedded in Durcupan (Fluka Chemie, Buchs, Switzerland). The polymerization occurred after 24 hours at 65°C.

The samples included were released from the outer casing and subsequently were placed under a stereo-microscope: with the help of a blade the excess resin has been removed to create around the piece a truncated pyramid, whose sides create angle of 45 ° with respect to the vertical axis and whose upper face is a regular trapezium. With an ultramicrotome (Leica Reichert Om-U3) were cut sections, i.e. sections of 0.5-2 μM thickness; sectioning was realized with an ultramicrotome characterized by glass blades and bowl containing double-distilled water on which the slices were released directly from the edge of the blade. Sections were colored with Toluidine blue 0.5% sodium carbonate and examined under light microscope to check the presence of the cells.

After the observation, a further reduction of the pyramid was done, in order to keep only the area of actual interest. The cutting operation is performed with diamond blade (dEYEmond), obtaining connected ultrathin sections settled on the surface of the water; color assumed by the ultrathin sections is a control of thickness: yellow-gold slices are of a thickness of 700-900 Å (70-90 nm), silver equal to 500-700 Å, gray to 400-500 Å. It is preferred silver-light yellow sections (600-800 Å) because allow good slice thickness and good contrast in TEM. Before harvesting, slices were subjected for a few seconds to vapors of xylene. Collection was performed on appropriate slotted copper disks of 3 mm in diameter whose perforated surface allows both the support of the slices and passage of the electron beam within the electron microscope.

The staining of the slices was made by the treatment of heavy metal salt solutions. These salts substances settled in differential mode on the sample, increasing the power of electronic dispersion of certain biological structures, and therefore the contrast between more or less electrondense areas.

Double staining was carried out first with 3% uranyl acetate in 50° alcohol for 5 minutes and subsequently with subnitrate of bismunto for 10 minutes. Staining was performed by inverting each screen on a drop of dye and washings must be extremely accurate in order to avoid contamination of the sections with precipitation of products of the dye solutions.

Ultrathin stained sections were observed by a JEOL (JEM 2200 FS) transmission electron microscope operated at 80 KeV (Tokyo, Japan). [108] [109] [95] Quantitative morphometry based on transmission electron microscopy analysis was performed to the detect interlamellar periodicity. The images were analyzed with ImageJ; images obtained by transmission electron microscopy where imported and several measurements were performed. [108]

### 6.2.5. Biochemical analysis

Biochemical analyses were performed at Department of Medical Biotechnology and Translational Medicine of University of Milan

#### 6.2.5.1. Lipid extraction

During the necropsy, frozen sample (stored at -185 C°, 2-3 g) of pathological brains, were collected from cortex, thalamus, medulla oblongata, and cerebellum; frozen samples (stored at -185 C°, 2-3 g), from the same areas, were later collected from age and sex matched animals. Samples were thawed on ice (4°C). Meninges were removed and sections were minced with a surgical blade, resuspended in ice-cold water and subjected to sonication. The samples were then Dounce homogenized (10 strokes, tight) before being snap frozen and subsequently lyophilized.

Lipids from the lyophilized samples were extracted with chloroform/methanol/water 20:10:1 (v/v/v) and subjected to a modified two-phase Folch's partitioning to obtain the aqueous (Aq. Ph.) and the organic phases (Or. Ph.). Briefly, 1550 µL of the solvent system were added to the lyophilized samples. The samples were then mixed at 1100 rpm, RT for 15 minutes and centrifuged at 13200 rpm, RT for 15 minutes. The supernatant was

collected as Total lipid extract (TLE) and the extraction was repeated again twice by adding the 1550 µL of the solvent system to the pellets. The pellets were air dried and resuspended in 1N NaOH and incubated overnight at RT before being with water to 0.05N NaOH to allow the determination of the protein content with DC assay. Aliquots of the TLE were then subjected to phase partitioning adding either 20% of water by volume or 20% of 0.88% KCl in $H_2O$ by volume. The samples were then mixed at 1100 rpm, RT for 15 minutes and centrifuged at 13200 rpm, RT for 15 minutes. The Aq. Ph. were recovered, and $CH_3OH$:$H_2O$ 1:1 (v/v) or $CH_3OH$: 0.88% KCl 1:1 (v/v) were added to the organic phase before mixing the samples at 1100 rpm, RT for 15 minutes and centrifuging at 13200 rpm, RT for 15 minutes. The new aqueous phases were recovered and united to the ones previously collected. The aqueous phases were dried under $N_2$ flux, and resuspended in water before undergoing dialysis and lyophilization. The organic phases were dried under $N_2$ flux and resuspended in a known volume of cholesterol/methanol 2:1. Aliquots of the organic phases were then subjected to alkali treatment to remove glycerophospholipids. [110]

#### 6.2.5.2. Thin layer chromatography (TLC)

To determine endogenous lipid content, the various samples were analyzed by mono-dimensional silica gel high performance thin layer chromatography (HPTLC) using different solvent systems. The total lipid extracts were analyzed using chloroform/methanol/0.2% aqueous 60:35:8 (v/v/v) as a solvent system, the aqueous phases were analyzed with chloroform/methanol/0.2% aqueous $CaCl_2$ 50:42:11 (v/v/v), whereas the organic phases and the methanolyzed organic phases were analyzed using chloroform/methanol/water 110:40:6 (v/v/v).

After separation, lipids were detected either by spraying the TLC plates with different colorimetric reagents (anisaldehyde, Ehrlich's reagent) or by TLC immunostaining. Identification of lipids after separation and chemical detection was assessed by co-migration with lipid standards [110]

## 6.3. Results

### 6.3.1. Gross lesions

The first animal necropsy was performed by Istituto Zooprofilattico of Lombardy and Emilia Romagna, while 2 remaining were performed at Operative Unit of Veterinary Pathology of University of Parma.

Gross lesions were scant and similar in all 3 animals with:

- Severe diffuse hepatic degeneration;
- Severe diffuse gastric and intestinal dilation;
- Brain was characterized by moderate meningeal congestion and reduction in consistency of cerebral and cerebellar parenchyma.

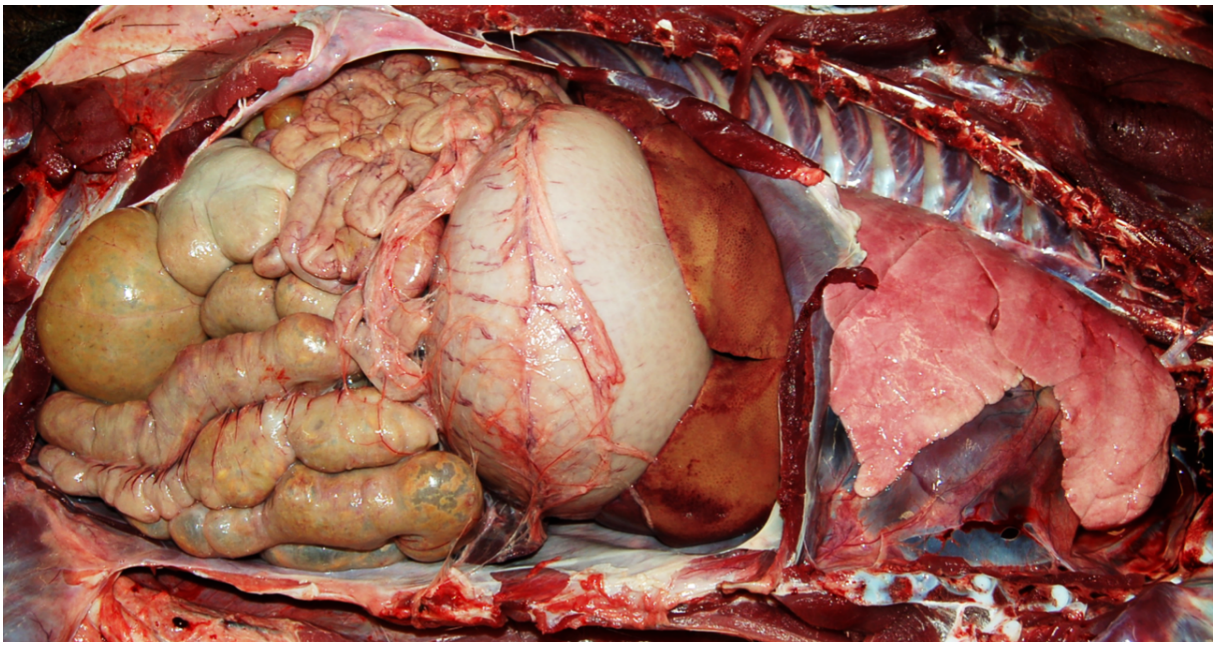


*Fig. 12 Gross appearance of thorax and abdomen. There is severe diffuse hepatic degeneration, gastric and intestinal dilation.*

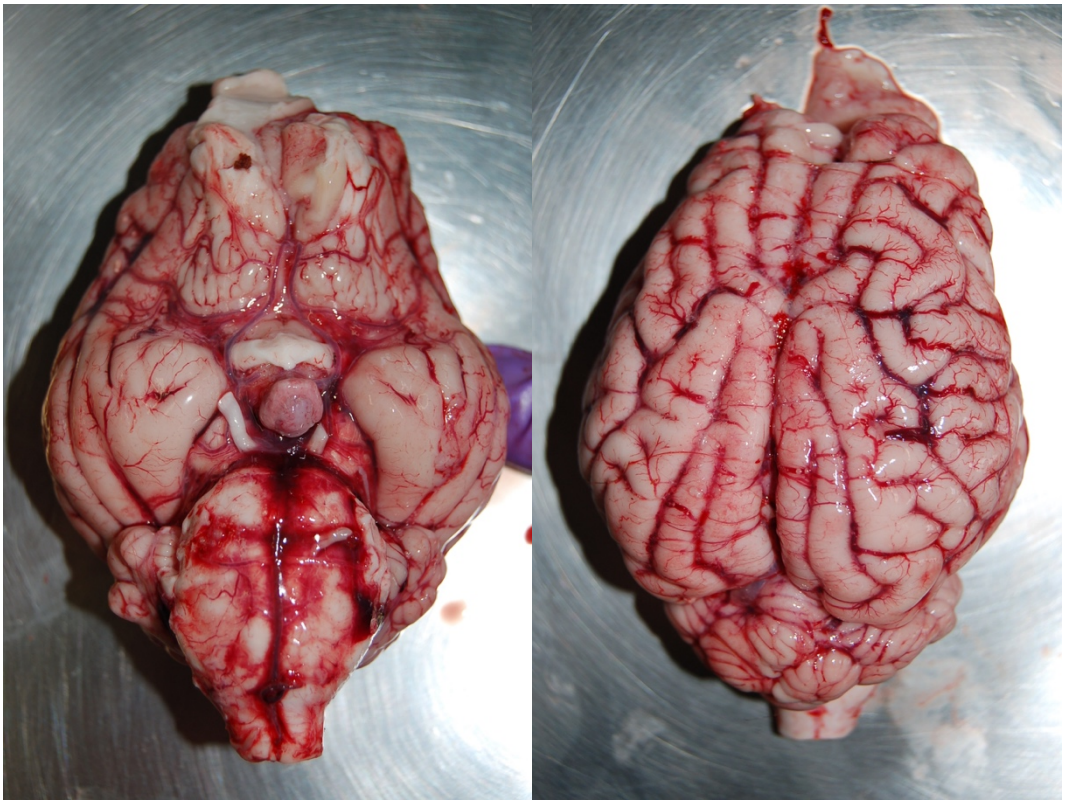


*Fig. 13 Gross appearance of the brain of the female boar*

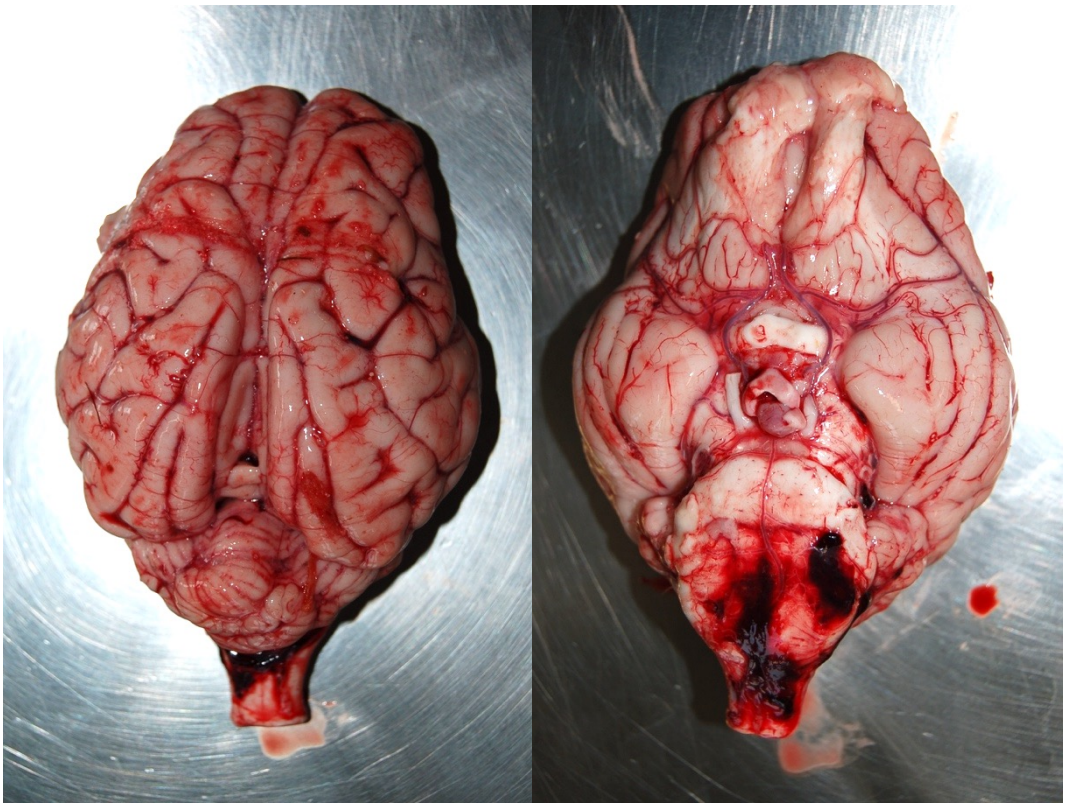


*Fig. 14 Gross appearance of the brain of the male boar*

### 6.3.2. Histology

- **Brain and cerebellum:** On histologic examination of formalin-fixed paraffin-embedded (FFPE) of all brain sections, grey matter of all the cerebral sections, nuclei and Purkinje layer is diffusely expanded by an abundant number of variably enlarged neuronal bodies. Enlarged neurons (up to 50 microns in diameter) have a rounded and distinct cell outline, abundant finely faintly eosinophilic granular to foamy vacuolated cytoplasm, with peripherally displaced oval and vesicular nuclei; vacuolizations are variably in size, ranging from 2-3 micron up to 20-30 micron, and filled by pale eosinophilic granular material or optically empty. Multifocally nuclei are shrunken, hyperchromatic and crescent shaped with curse chromatin (pyknotic) or moved from to the center to the plasma membrane with effacement of Nissl substance (central chromatolisis).

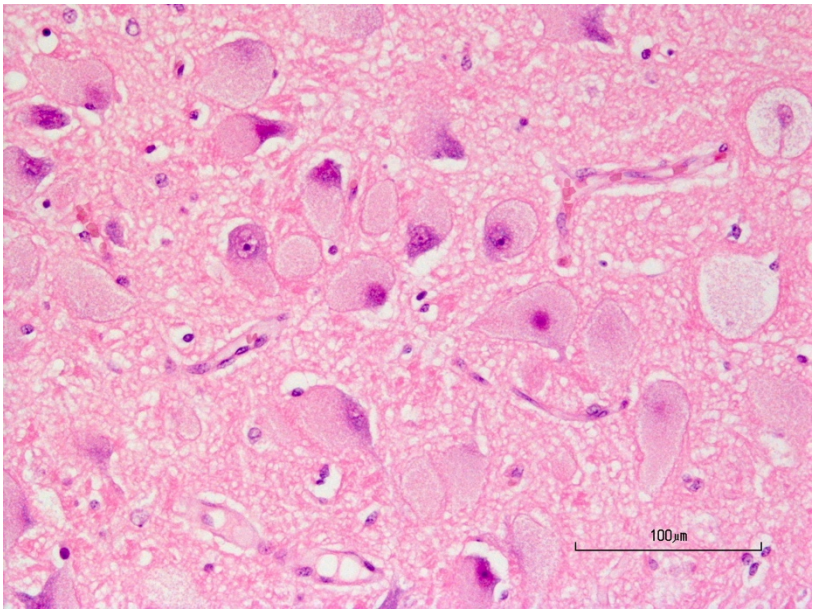



*Fig. 15 Brain, Cortex. Neurons are severely enlarged and have finely faintly eosinophilic granular to foamy vacuolated cytoplasm; furthermore, multifocally are characterized by central chromatolisis H&E, bar 100 µm*

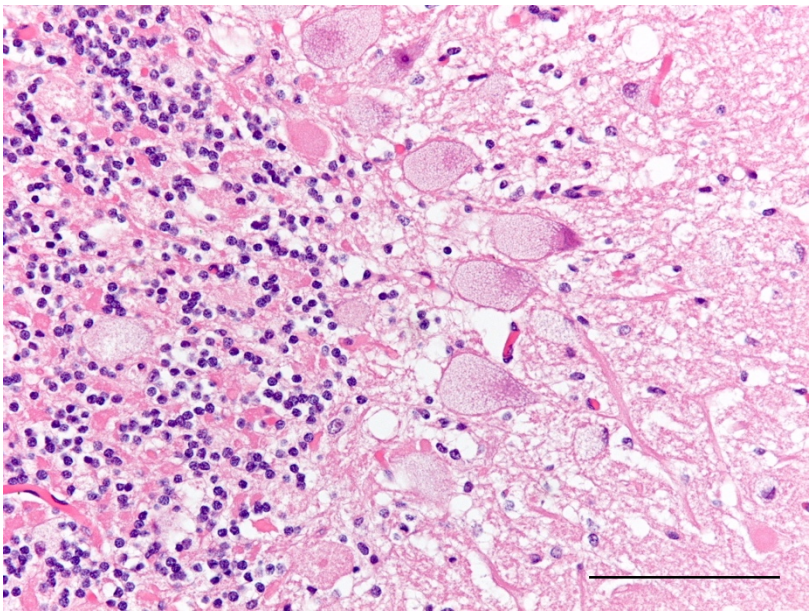


*Fig. 16 Cerebellum. Purkinje layer is diffusely expanded by an abundant number of variably enlarged neuronal bodies. H&E, bar 100 μm*

Multifocally, the axon hillock of neurons may also be hypertrophied to accommodate the stored material, forming a meganeurite.

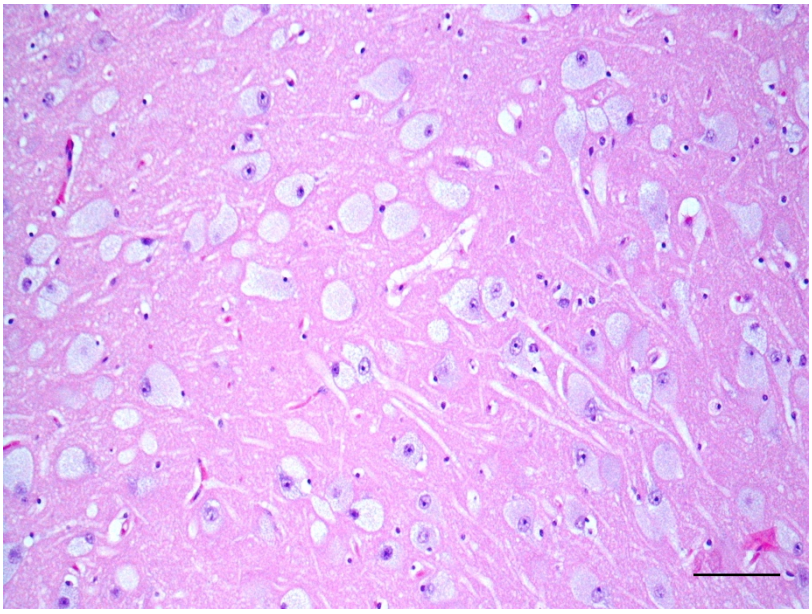


*Fig. 17 Brain, cortex. Axon hillock of neurons are enlarged by storage material (Meganeurites). H&E bar 500μm*

Intermingled with neuronal nuclear groups, scattered within both the surrounding white and gray matter and intermingled in the granular layer of the cerebellum, there are numerous round swollen axons (spheroids) with variable loosely granular to densely eosinophilic appearance, raging in diameter from 10 to 30 μm.

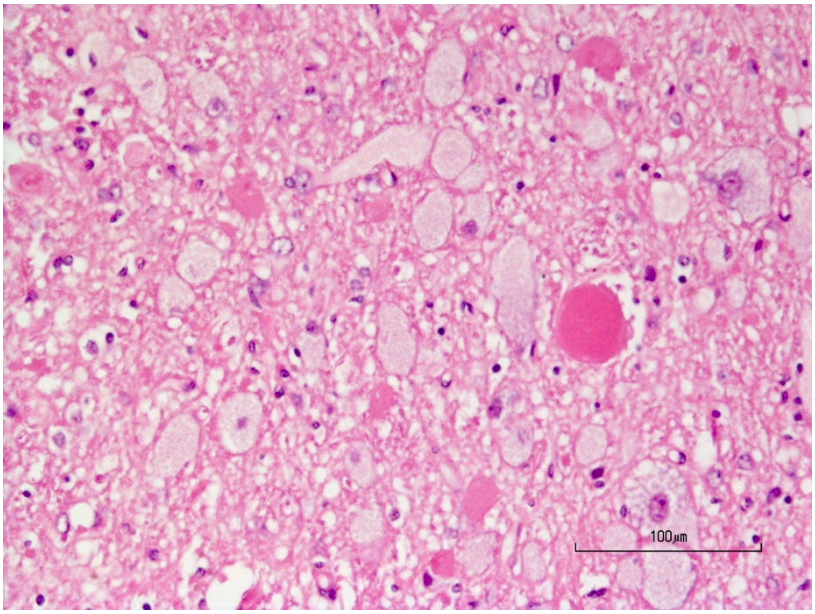



*Fig. 18 Brain, cortex. Presence of several round swollen axons (spheroids). H&E, bar 100 μm*

Multifocally Purkinje cell dendrites are thickened.

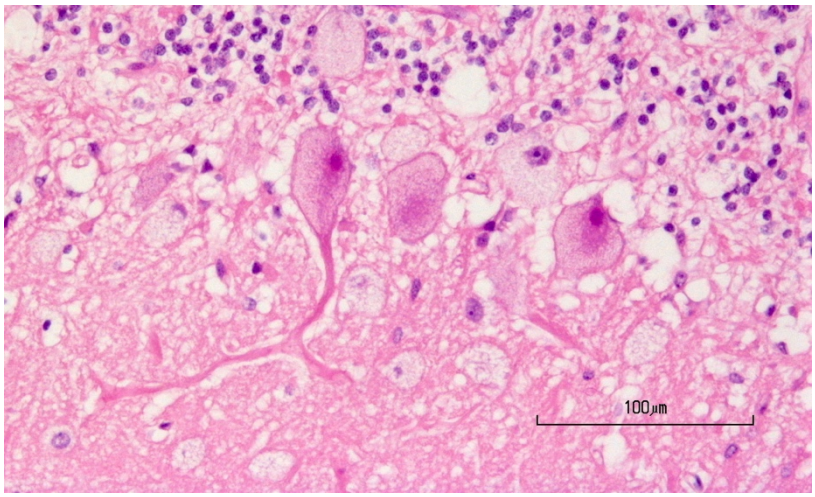



*Fig. 19 Cerebellum. Thickened Purkinje cell dendrites. H&E, bar 100 μm*

Elevate number of glial cells are diffusely present throughout the cerebral parenchyma: these are multifocally increased in number (astrocytosis) with hypereosinophilic cytoplasms and oval vesicular nucleus (gemistocyte); increase in number astrocytic nuclei with conspicuous cytoplasm are defined as reactive gliosis.

Multifocally, adjacent to degenerated neurons, there are occasionally macrophagic cells (gitter cells) with foamy or eosinophilic granular cytoplasm (stored material) or perineuronal satellite oligodendroglial cells are characterized by hypertrophy and hyperplasia (satellitosis).

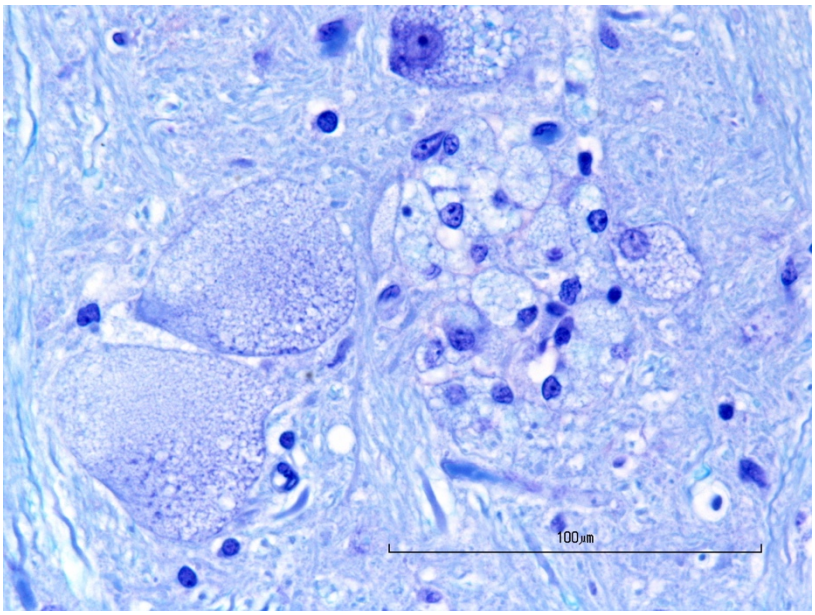



*Fig. 20 Brain, medulla oblongata. Severely enlarged neuronal bodies and gitter cells with foamy cytoplasm (stored material). Luxol fast blue, bar 100 µm*

Multifocal there are small areas (50-100 micron) characterized by loss of cerebral parenchyma with rarefaction and glial cells (malacia).

Virchow–Robin space are frequently evident and increased by clear space (edema).

- **Spinal cord**: ventral and dorsal horn are characterized by neurons enlarged and swollen with the same cytoplasmic storage. There is multifocally gliosis and astrocytosis.

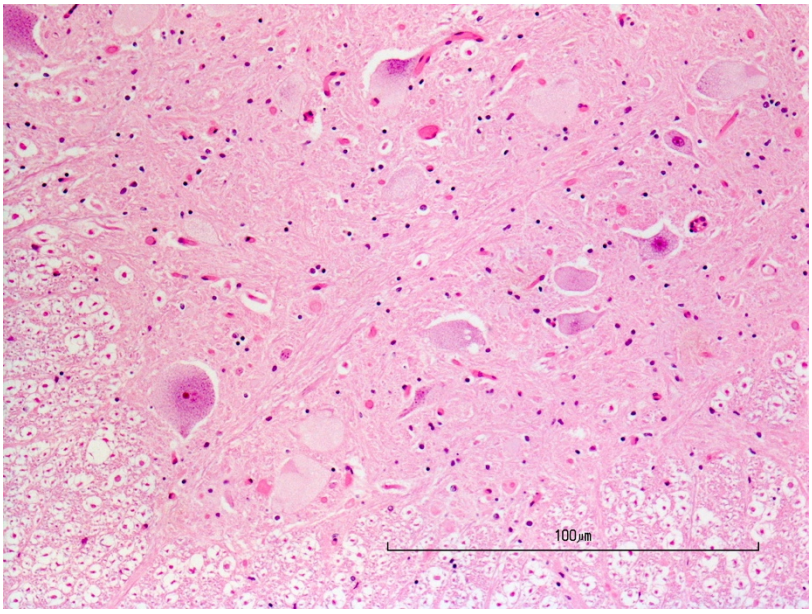



*Fig. 21 Spinal cord. Horns are characterized by pathological neurons; there is moderate astrocytosis. H&E, bar 100 µm*

- **Peripheral ganglia:** peripheral (nerve and autonomic) nervous system are characterized by rounded neurons with cytoplasmic vacuolization.

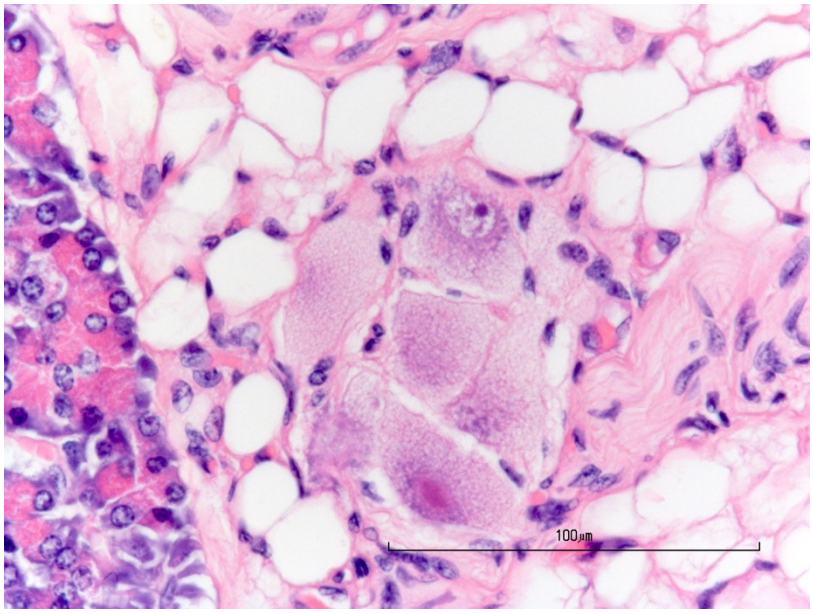



*Fig. 22 Pancreas. Peripheral ganglia with pathological neurons. H&E, bar 100 µm*

- **Retina:** Retinal ganglion cells are affected by the aforementioned cytoplasmic vacuolation

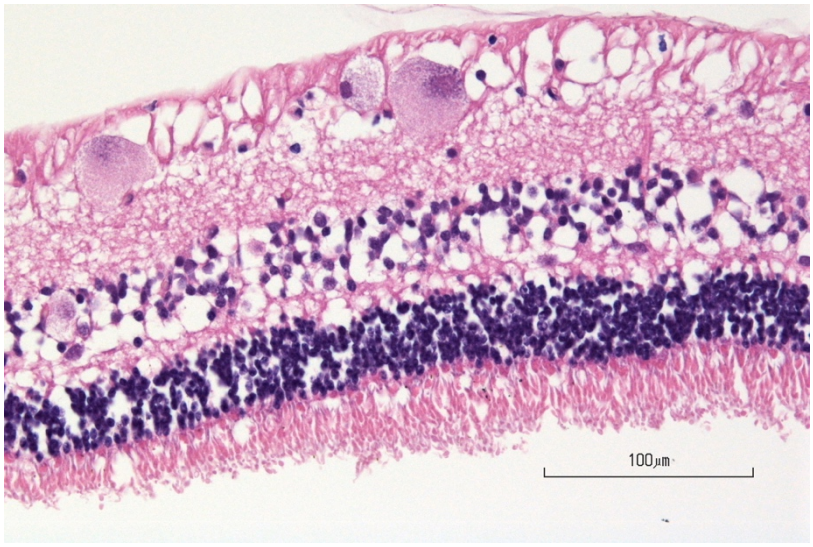



*Fig. 23 Retina. Retinal ganglion cells with enlarge foamy appearance. H&E, bar 100 μm*

- **Liver:** Hepatocytes and Kupffer cells are characterized by the same finely diffusely vacuolated cytoplasm. Numerous hepatic stellate cells (pericytes) are present and there is moderate multifocal bile stasis.

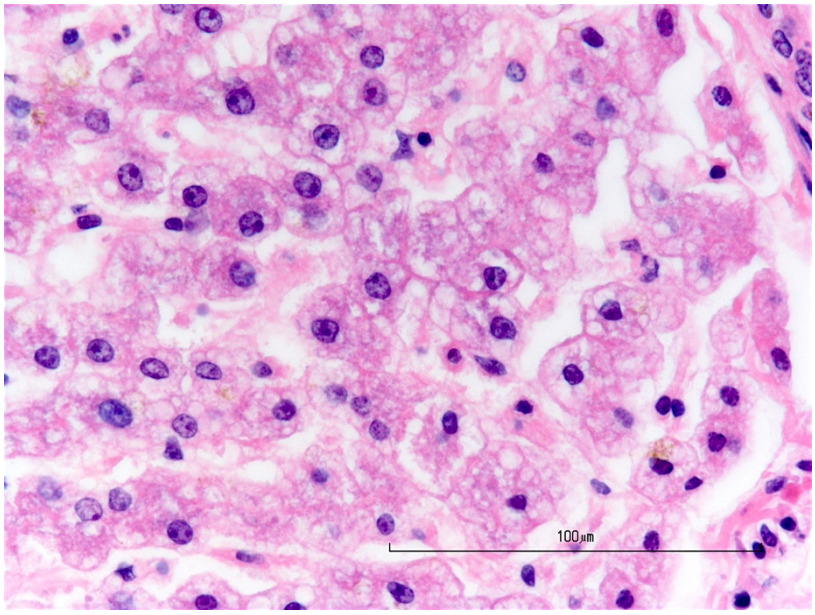



*Fig. 24 Liver. Hepaocutes are charcterized by foamy vacuoleted appearence. H&E, bar 100 μm*

- **Other organs**: are characterized by diffusely congestion.

### 6.3.3. Histochemical and Immunohistochemical stainings

- **Histochemical stainings:** Periodic acid-Schiff (PAS), Toluidine blue and Luxol Fast blue were performed on different sections of brain and spinal cord. Cytoplasmic neuronal vacuolation resulted variably positive for PAS and Luxol fast blue stainings in FFPE sections and diffusely positive for Toluidine blue in resin-embedded sections.

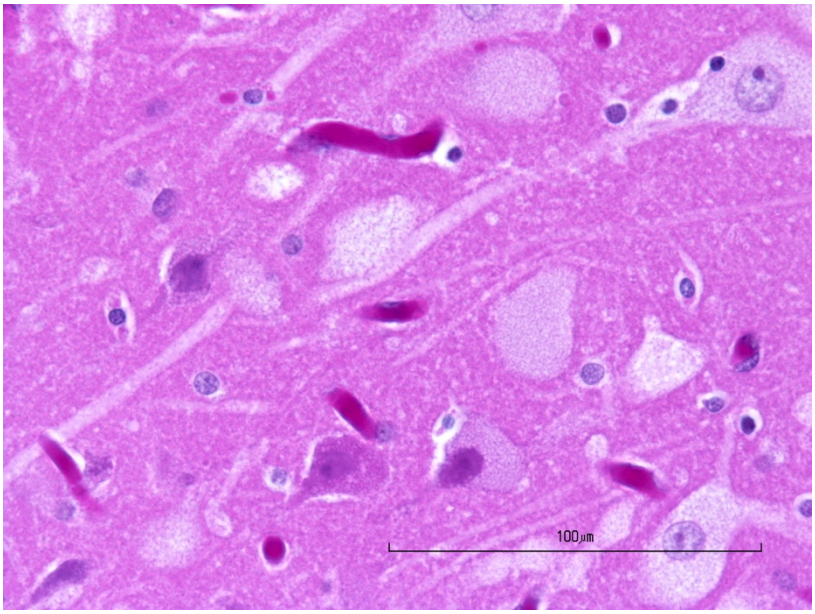



*Fig. 25 Brain, cortex. Neurons have cytoplasmic vacuolation with content that stains variably positive with PAS stain. PAS, bar 100 µm*

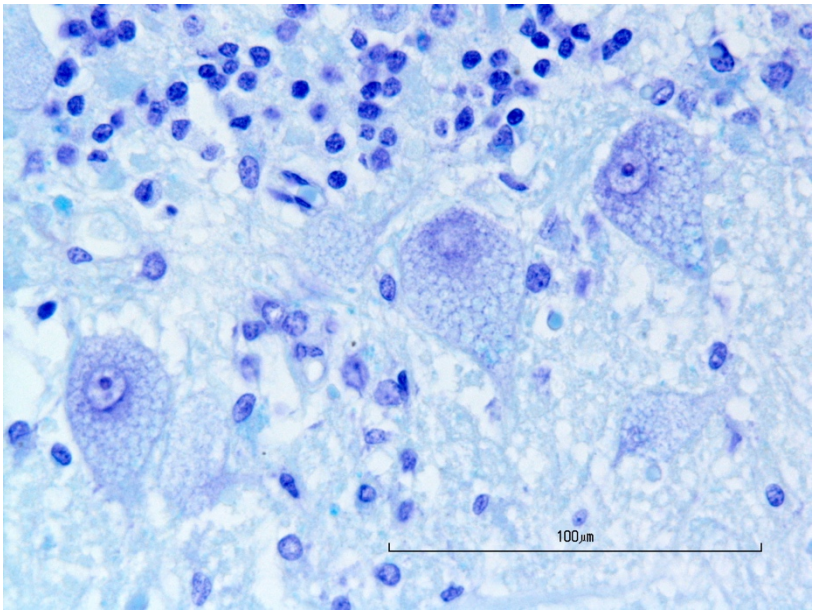



*Fig. 26 Cerebellum. Purkinje cell with stored cytoplasmic material that stain positively for Luxol fast blue. Luxol fast blue, bar 100 µm*

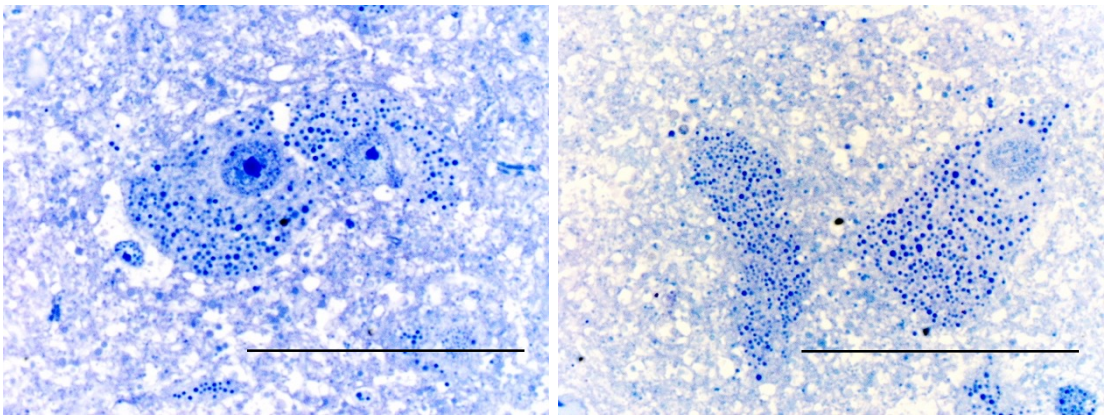


*Fig. 27 Brain, cortex. Cytoplasmic neuronal stored material results positive for Toluidine blue stain. Toluidine blue, resin-embedded sections, bar 100 μm*

PAS also denote the presence of numerous spheroids and loss of homogeneity of the neuropil.

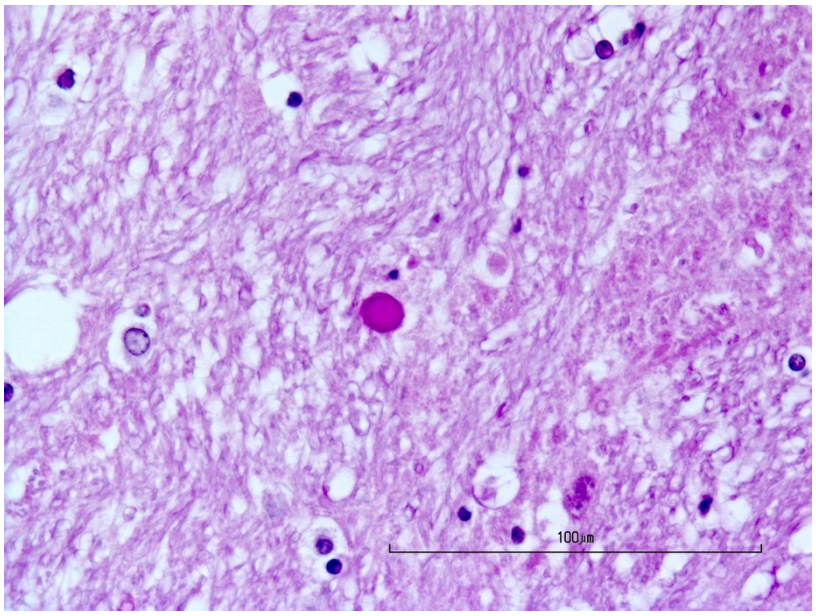



*Fig. 28 Brain, cortex. Spheroid stain positively for PAS stain. PAS, bar 100 μm*

Evaluation of the myelin distribution was performed using Luxol fast blue and is denote a reduction in dimension of white matter with loss of homogeneity in the distribution.

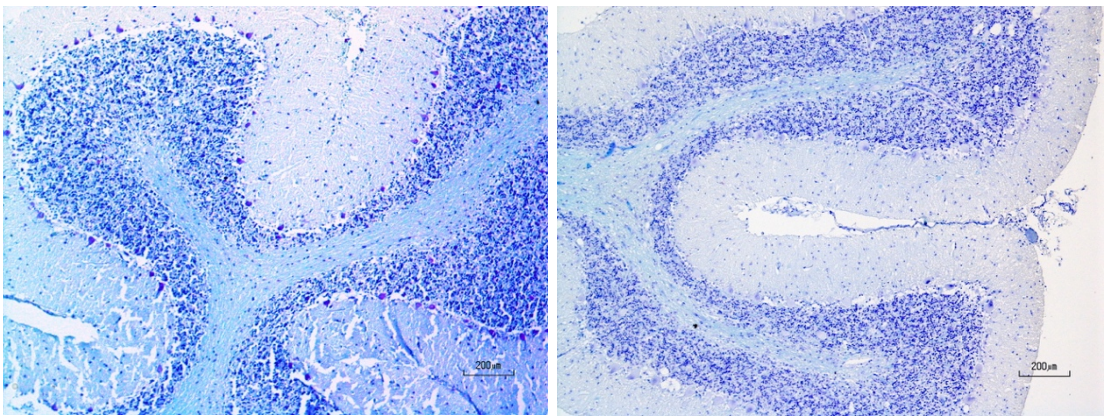


*Fig. 29 Cerebellum (normal left, pathological right). White matter is reduced in dimension and have an non homogeneous appearence. Luxol fast blue bar 200μm*

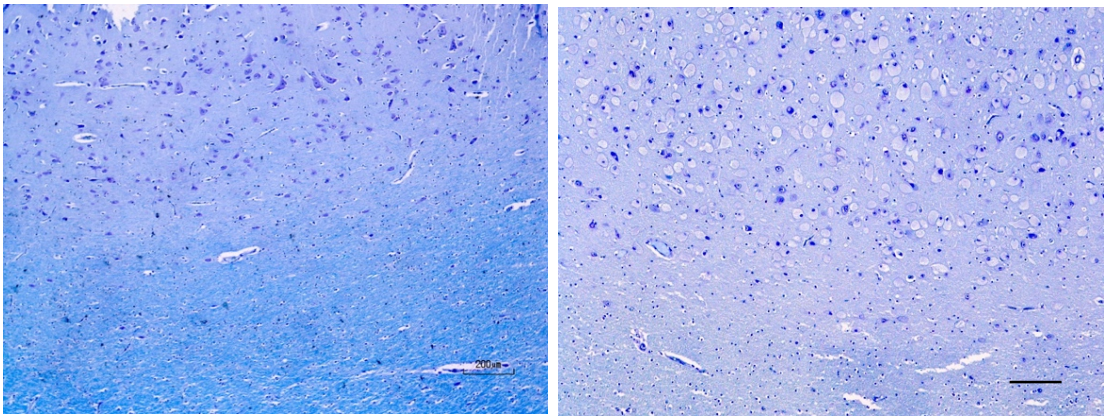


*Fig. 30 Brain, cortex Cerebellum (normal left, pathological right). White matter has severe reduction of myelinization. Luxol fast blue bar 200μm*

- **Immunohistochemical stainings**: IHC for GFAP demonstrate in affected animals, compared to normal ones, astrocytes with up regulated expression of the marker and exhibit cellular hypertrophy, but with preservation of individual astrocyte domains and without pronounced overlap of astrocyte processes; some areas are characterized by severe diffuse reactive astrogliosis with pronounced up regulation of GFAP expression, astrocyte hypertrophy, astrocyte proliferation and pronounced overlap of astrocyte processes resulting in disruption of individual astrocyte domains.

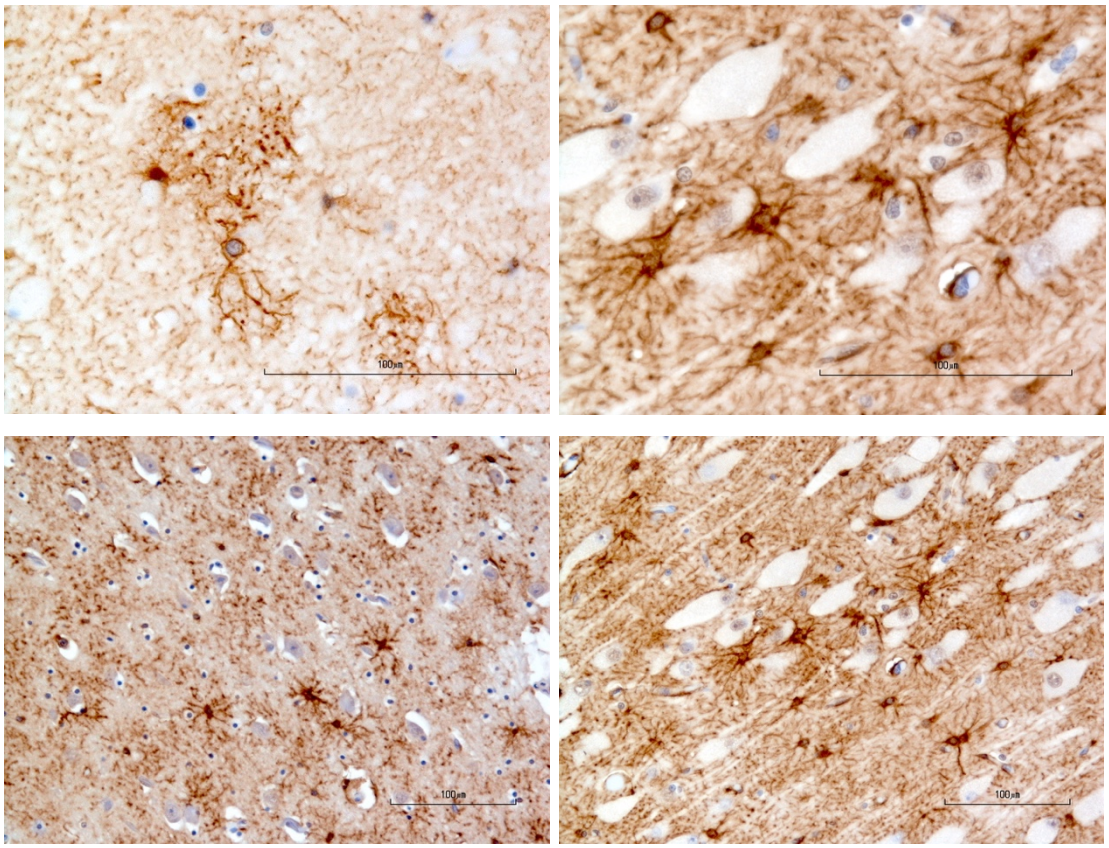


*Fig. 31 Brain, cortex (normal left, pathological right). Affected brains are characterized by astrocyte proliferation, with hypertrophy and overlap of processes. IHC for GFAP, bar 100 μm*

IHC for Vimentin demonstrate in affected animals, compared to normal ones, the presence of numerous positive astrocytes; these are frequently intermingled with cortical neurons, uniformly distributed or forming small aggregates within particularly the white matter.

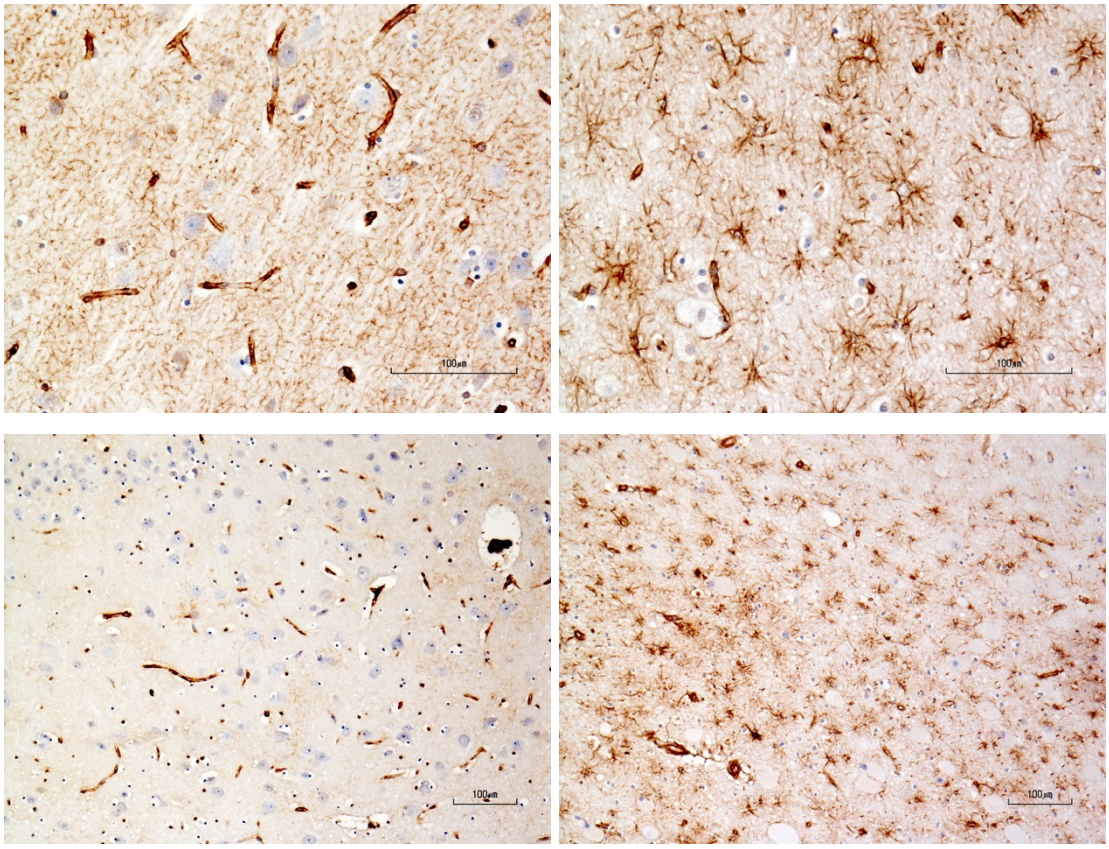


*Fig. 32 Brain, cortex (normal left, pathological right). Affected brains are characterized by severe astrocyte proliferation and overlap of processes. IHC for Vimentin, bar 100 μm*

### 6.3.4. Transmission Electron Microscopy (TEM)

Transmission electron microscopy was performed only on cerebral cortex and reveals at low magnification the presence of numerous swollen and degenerated neurons; these are diffusely characterized by discontinuous cellular membrane and multifocally loss of distinction of cellular margins. Cytoplasm are severely enlarged and filled by single lysosomes, that frequently form large aggregates, filled by membranous material. These structures are defined as “Membranous cytoplasmic bodies”, which are characterized by membranous material single or multiple, layers of concentric outer membranes surrounding inner components of short, straight or curved membranes. Dimension of membranous bodies varies from 0.6 up to 1.0 μm and average interlamellar periodicity is 33 $\pm$ 1 nm.

Cytoplasm is also characterized by the presence of numerous large, round, double membrane bound, swollen mitochondria with reduced electrondensity of the matrix and loss of mitochondrial cristae (mitochondrial swelling and cristolysis). Scattered within the cytoplasm, is detectable Golgi complex, composed of irregular, occasionally swollen, dilated, stacks and tubules lined by a single membrane and associated with high number of small, round, single membrane-bound empty vacuoles and vesicles; these are occasionally disrupted and filled with granular grey material. Few, disrupted, tubular elements of rough endoplasmic reticulum are detectable, while variable number of ribosomes are distributed within the cytoplasm.

Nucleus is characterized by discontinuous nuclear membrane and contains multiple scattered aggregates of electrondense heterochromatin along the nuclear envelope (chromatin margination) and dispersed granular electronlucent euchromatin.

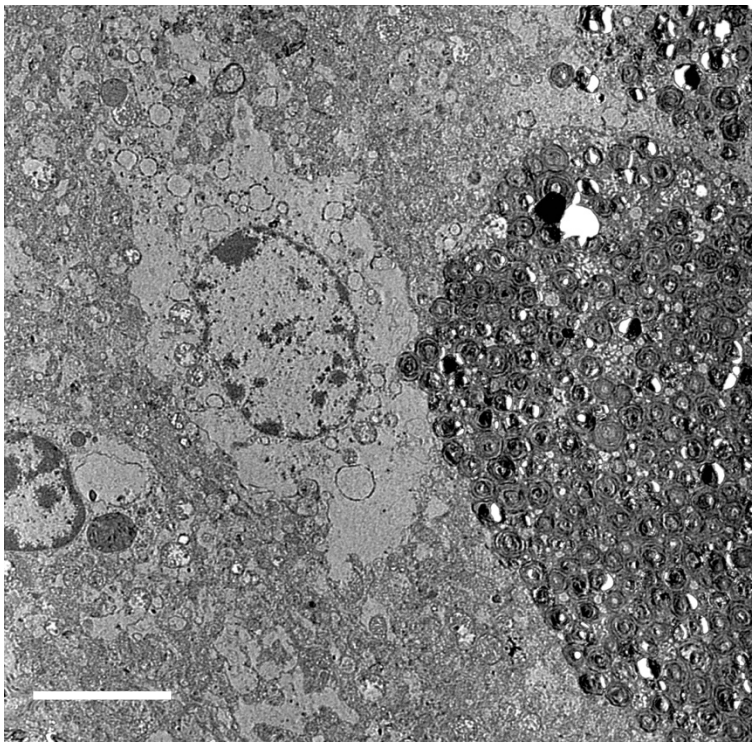


*Fig. 33 Brain, cortex. Portion of a neuronal body. Cytoplasm is partially filled by a large aggregate of membranous cytoplasmic bodies; several degenerated mitochondria are present. Nucleus is characterized by chromatin margination. TEM, bar 5μm*

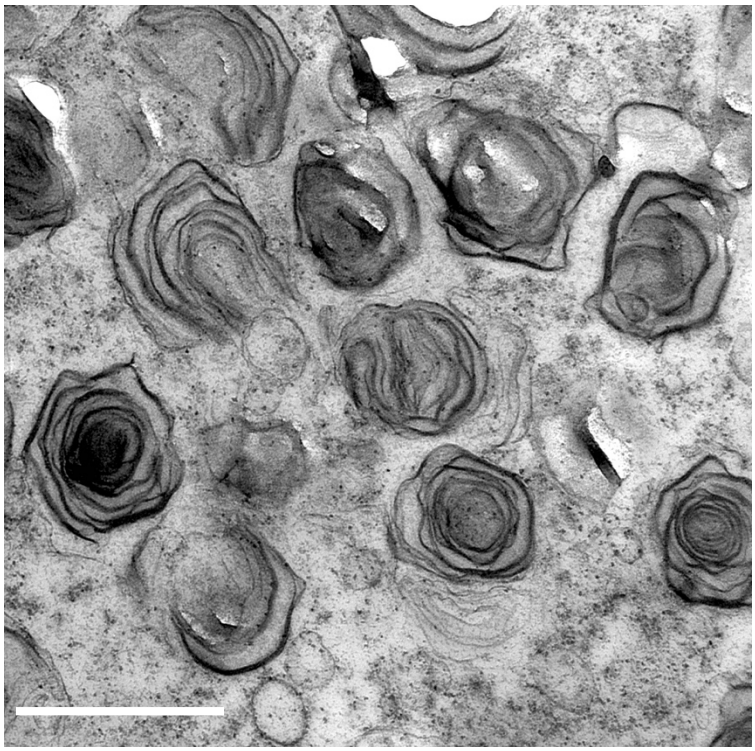


*Fig. 34 Brain, cortex. Membranous cytoplasmic bodies, arranged in aggregate, composed by several parallel or concentric layers of membranous material, with variable interlamellar periodicity. TEM, bar 1 μm*

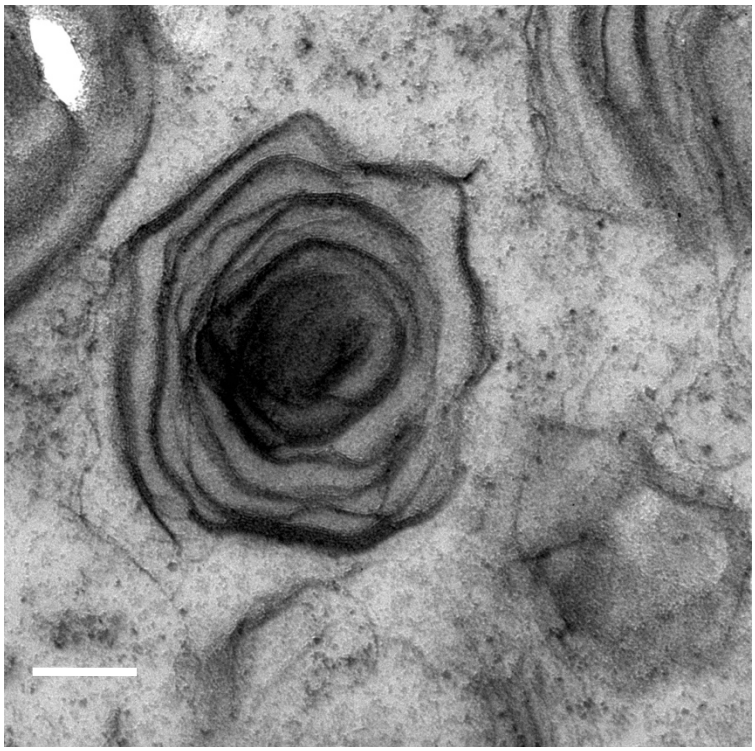


*Fig. 35 Brain, cortex. Membranous cytoplasmic body composed by single layer of membranous material, with elevate interlamellar periodicity. TEM, bar 200 nm*

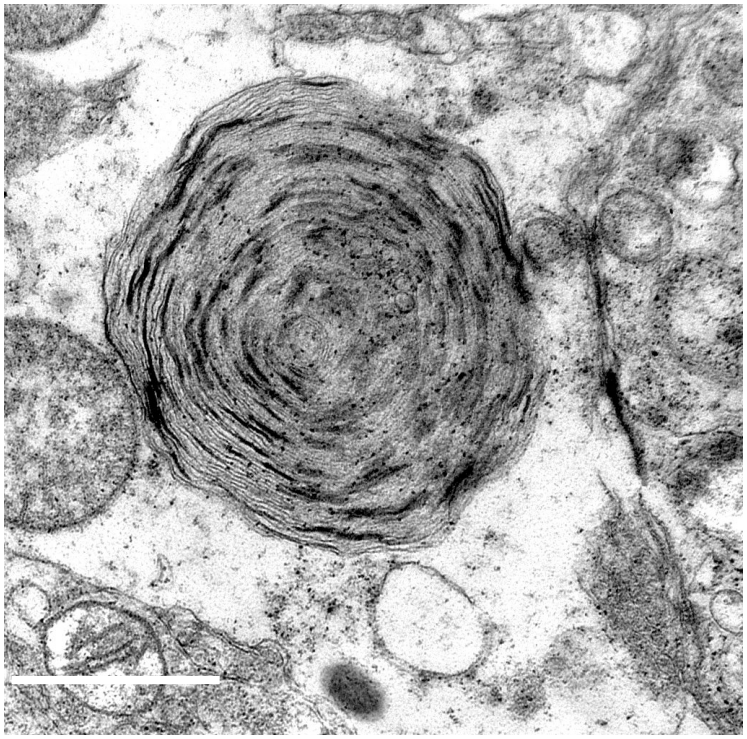


*Fig. 36 Brain, cortex. Membranous cytoplasmic body composed by numerous layer of membranous material with low interlamellar periodicity. TEM, bar 1 μm*

### 6.3.5. Biochemical analysis

Frozen samples from pathological brains, were collected from cortex, thalamus, medulla oblongata, and cerebellum to evaluate the lipid content. Total lipid portion was extracted from tissues, using organic solvents, and was separated to obtain aqueous and organic phases. The aqueous phase contains gangliosides (in virtue of their negative charge) and traces of neutral sphingolipids. The organic phase originally contains neutral glycosphingolipids and phospholipids. It is treated with alkali to hydrolyze the phospholipids and subjected to subdivision to eliminate the water-soluble part. It remains an organic phase which contains only a few sphingolipids and lipid contaminant.
The aqueous phase and the organic phase, containing sphingolipids, are analyzed with thin layer chromatography (TLC); posing amount of material on TLC corresponds to equal amounts of tissue proteins.

- **Aqueous phase**

  The figure underline that, in normal tissues, (8. Cortex, 11. Thalamus, 13. Medulla oblongata and 16. Cerebellum) there was absence of ganglioside GM2 (see line 22 for standard) and ganglioside GMN2 is an extremely minority component in the normal brain.

  In lipid portion from pathological animals (9 and 10. Cortex, 12. Thalamus, 14. Medulla oblongata, 17. Cerebellum and 18. Hypothalamus), GM2 ganglioside was a major component of the aqueous phase.

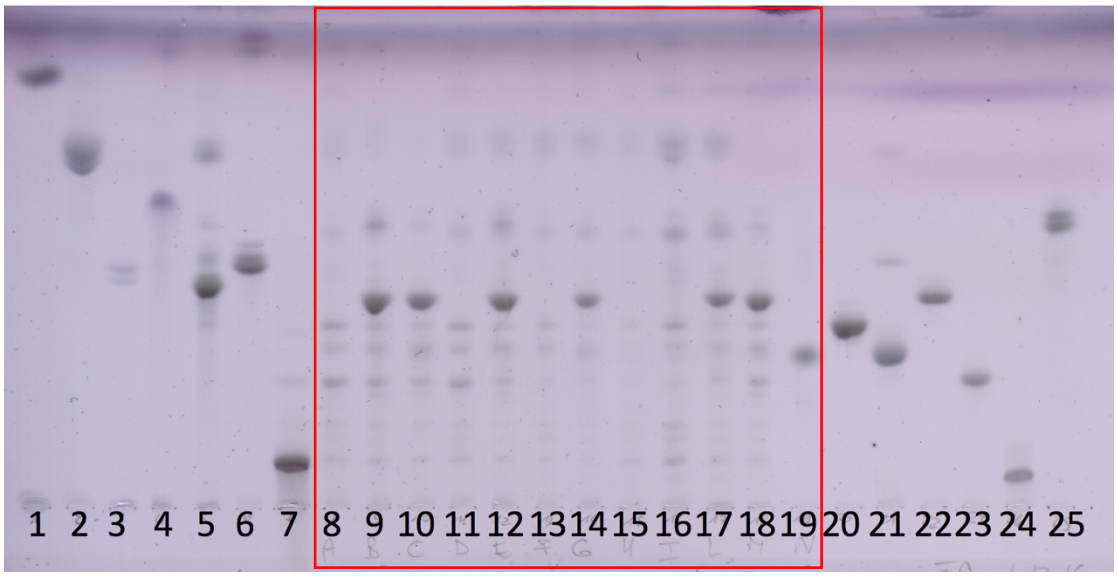
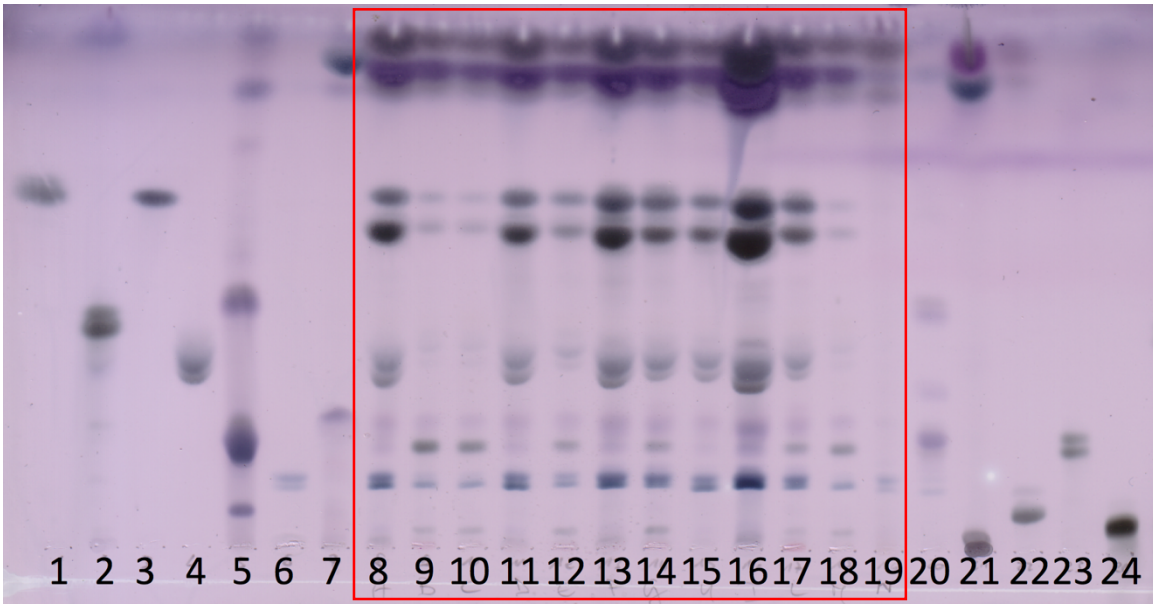



*Fig. 37 Mono-dimensional silica gel high performance thin layer chromatography (HPTLC) of the aqueous phase of total lipid content of normal (wt)and pathological (Pat) brain of wild boars*

1. *Galactosylceramide*
2. *Sulfatide*
3. *Sphingomyelin*
4. *Phosphatidic acid*
5. *Asialo GM1*
6. *GM3*
7. *GT1b*
8. *Cortex wt*
9. *Cortex Pat.*
10. *Cortex Pat.*
11. *Thalamus wt*
12. *Thalamus Pat.*
13. *Medulla oblongata wt*
14. *Medulla oblongata Pat.*
15. *Cerebellum A wt*
16. *Cerebellum B wt*
17. *Cerebellum Pat.*
18. *Hypothalamus Pat.*
19. *Liver Wt*
20. *GM1*
21. *GD3*
22. *GM2*
23. *GD1a*
24. *GQ1b*
25. *Triesosil ceramide*

- **Organic phase**

  In pathological tissues, there was a drastic reduction of principal components of myelin: sphingomyelin (6), sulphatide (4) and cerebrosides (present as doublets due to the heterogeneity of the lipid chains, hydroxylated and non-hydroxylated). Furthermore, is evident an increase in triesosilceramide portion.




- *Fig. 38 Mono-dimensional silica gel high performance thin layer chromatography (HPTLC) of the organic phase of lipid content of normal (wt)and pathological brain of wild boars (Pat)*

1. *Glucosylceramide*
2. *Lactosylceramide*
3. *Galactosylceramide*
4. *Sulfatide*
5. *Cephalin (PE)+ Lecithin (PC)*
6. *Sphingomyelin*
7. *Ceramide C24 and phosphatidic acid*
8. *Cortex wt*
9. *Cortex Pat.*
10. *Cortex Pat.*
11. *Thalamus wt*
12. *Thalamus Pat.*
13. *Medulla oblongata wt*
14. *Medulla oblongata Pat.*
15. *Cerebellum A wt*
16. *Cerebellum B wt*
17. *Cerebellum Pat.*
18. *Hypothalamus Pat.*
19. *Liver Wt*
20. *Phospholipids mixture*
21. *Ceramide C16*
22. *GM3*
23. *Triesosil ceramide*
24. *Tetraesosil ceramide*

# 7. Discussion: comparison between human and wild boar pathology

Lysosomes are cellular organelles whose primary function is to break down macromolecules for export or recycling, and they are now thought to play a significant role in the regulation and maintenance of cellular homeostasis.
Lysosome dysfunction is the direct cause of the pathogenesis of LSDs. In LSDs, mutations in lysosomal hydrolases or transporters result in the accumulation of specific macromolecules, leading to progressive reduction in the capacity of the lysosome to respond to cellular queues and normal processing, which, in turn, leads to secondary changes in cellular events, such as impairment in autophagy, mitochondrial dysfunction, inflammation, and cell and tissue death. Even though the defective proteins in LSDs are found in multiple cell and tissue types, there is selective vulnerability in the extent to which these lysosomal defects affect the functioning and viability of each cell type. [3]
Gangliosides play a number of important roles in brain development and function, including receptor-mediated cell signaling, ion channel modulation, and dendritogenesis. They are found in the neuronal plasmalemma, where they co-localize with other glycosphingolipids and cholesterol in specialized areas of the membrane known as lipid domains or rafts. [84] Gangliosides accumulates within lysosomes due to the defects in the genes coding for 2 possible enzymes, β-galactosidase and Hexosaminidase, causing respectively GM1 and GM2 gangliosidosis.
The clinical and pathological findings of GM2 gangliosidosis in wild boards are very similar, but not completely identical, to those described in human GM2 gangliosidosis; this can be caused by the relatively limited duration of the boar disease, that were euthanatized in the course of their disease.
Clinically GM2-gangliosidosis can be sub-classified into infantile, juvenile, chronic, and adult onset forms; particularly children affected by the infantile form are normal at birth and show first symptoms, such as mild weakness between 3

and 6 months of life. Decreased motor activity, blindness, an exaggerated extensor response to sound, and eventual seizures and obtundation, are progressive clinical signs. A finding in most cases is a cherry red spot in the central retina of the patients, particularly in Tay-Sachs disease. [4]

In the first 12 to 14 months of the human disease, gross pathologic findings include brain atrophy, widening of the sulci, and mildly dilated ventricles. Between 15 and 24 months, the brain often increases in weight with enlargement of the cerebrum and atrophy of the cerebellum and brain stem; brains are firm in consistency, are rubbery, and lack a clear distinction between cerebral gray and white matter. [111] Brains of wild boars were grossly characterized only by reduction in consistency of cerebral and cerebellar parenchyma, more similar of the “early” manifestations.

The histological findings in brain of wild boards were very similar to those described in human GM2 gangliosidosis, with diffusely neurons throughout the central and peripheral nervous systems enlarged with round to ovoid shape, distended by foamy to granular cytoplasm. The cytoplasmic stored material stained positively with Luxol fast blue in FFPE, Toluidine blue in resin-embedded and resulted weakly positive with PAS in FFPE; PAS weakly and variable positivity is caused by removal during fixation and paraffin-embedding of the uncatabolized substrate that has accumulated in lysosomes, resulting positive in frozen tissues.

Other neuropathological changes are similar in wild boars and human GM2 Gangliosidosis.

Spheroids are swellings of significant size that occur along the length of an axon with axonal continuities clearly visible on both proximal and distal sides; they are caused by the accumulation of different specific organelles (mitochondria, membrane-bound vesicles, dense bodies). They are characteristically associated with GABAergic neurons, particularly in gray matter areas; this is probably due to the higher firing rates and metabolic activity, resulting in a greater turnover in axonal and synaptosomal components, and thus greater

reliance on fully intact anterograde and/or retrograde transport mechanisms. Axoplasmic transport defect or deprivation of key transport molecules are the possible cause underling the formation of spheroid in lysosomal storage diseases. Furthermore, it has been demonstrated a striking correlation between the location and the incidence of axonal spheroids and the type and severity of clinical neurological disease, more than intraneuronal storage, meganeurite formation, ectopic connectivity and cell death. [112] [113]

Meganeurites, that are parasomatic enlargments within the axonal hillock, are secondary to storage, since it has been demonstrated that they contain storage cytosome. Meganeurites can be “spiny” (covered with dendritic-like spines and new synapse formation) or “aspiny” (lacked any evidence of dendritic spines or synapse formation); it has been demonstrated that specific type of neurons, in different storage disease, appeared capable of undergoing elaboration of new membrane occurring either as neuritic sprouts or as spiny meganeurites (or both). [113]

Ectopic dendritogenesis, defined as growing of ectopic dendrites, have been demonstrated on the same type of neurons, across diverse type of storage diseases: cortical pyramidal neurons and multipolar cells of the amygdala and claustrum, neurons with pyramidal-like morphology. Through a series of studies, has been established that GM2 ganglioside is a metabolic product consistently elevated in the cerebral cortex of all storage diseases characterized by ectopic dendritogenesis and the elevation appears before the dendritic spouting; these findings indicates that GM2 increasing can be a cause of ectopic dendritogenesis. [113]

All these pathological changes that affects the neuron can lead to neuronal death; in human patients and mouse models, this appears to be the result of apoptosis, probably triggered by an alteration in endoplasmic reticulum of calcium levels resulting in an unfolded protein response. [114] [15]

Myelin staining with Luxol fast blue stain was decreased and inhomogeneous in the cerebral and cerebellar white matter, compared with control tissue. The

causes of the decrease myelin observed in several human lysosomal storage diseases is still under investigation, but several studies have confirmed direct metabolic effects on oligodendrocytes contributing to delayed or abnormal myelination (dysmyelinogenesis). This results from a multiplicity of factors, likely reflecting both primary effect of the metabolic disease on normal oligodendroglial growth and function, as well as a secondary influence of aberrant neuroaxonal development on myelination. Furthermore, despite the difference in pattern and timing of myelination among the various mammalian species, comparisons to human development and pathogenesis are possible. [115]

Astrocytes are characterized by several immunohistochemical markers: in normal brain, Vimentin is the main intermediate filament proteins of immature astroglial cells, whereas maturing astrocytes express vimentin and adult astrocytes GFAP only. Furthermore, in normal brains, astrocytes processes do not overlap and many astrocytes do not express detectable levels of GFAP. GFAP and vimentin expression is also observed in activated astrocytes of reactive gliosis. [116] [117] [118] IHC performed on boar's brains, demonstrated elevate expression in astrocytes of GFAP; these were hypertrophied with diffusely preservation of individual astrocyte domains, rare areas of overlap of astrocyte processes and scattered areas of aggregation, particularly in cortical areas. These findings can be defined as mild to moderate reactive astrogliosis. [116] Astrocytic expression of Vimentin in pathological brain, compared to normal brain where is almost completely absent, is severely higher; moreover, Vimentin positive astrocytes are frequently intermingled with cortical vacuolated neurons.

Severe astrocytic expression of Vimentin has been interpreted as a transient reversion to an immature phenotype or as a recruitment of immature astrocytes. [119] [116] [120] Available evidence in human pathology, suggests that astrocyte activation is an early indicator of CNS pathology in LSD, which conceivably contributes to neurodegeneration in later stages of disease

progression. However, whether astrocyte dysfunction is a primary cause of neuronal loss or rather a consequence of reactivity to diseased neurons in the context of LSDs remains undetermined. Recent studies suggest that neurodegeneration in LSDs may be influenced by astrocyte dysfunction. However, it remains to be determined if astrocyte involvement in neuronal demise is a universal phenomenon across several LSDs or rather, is only applicable to select degenerative processes. [119] in mouse models, astrocytosis appears to involve the lipid-signaling molecule sphingosine-1-phosphate (SIP), as SD mice with targeted deletion of the SIP gene develop milder disease than mice with the functional gene. Is also suggested that the combined downregulation of phosphorylated Akt and upregulation of phosphorylated extracellular, signal-regulated kinase (ERK) may be responsible for the proliferation of astrocytes in SD mice. [121] [122]

Inflammation occurs in boars' pathology (mild) as in human, during disease development, although the role it plays in the pathogenesis is still unclear; in mouse models, it has been shown that activated microglia are present prior to the onset of neurodegeneration. Microglia in TSD and SD mice show enhanced MHC class II expression, evidence of nitric oxide formation, and elevations in inflammatory cytokines such as tumor necrosis factor, interleukin-1$\beta$, transforming growth factor-$\beta$1, and macrophage inflammatory protein-1$\alpha$. [123] [124] [119]

Transmission electron microscopy (TEM) revealed the intracytoplasmic presence of large number of membranous cytoplasmic bodies (MCB), Specific ultrastructural aspects of the lysosomal inclusions are characteristic for certain syndromes; particularly MCB are characteristic for Gangliosidosis. [108] These are membrane-bound lysosomes distended with membranous material arranged in concentric circles or lamellae. The core of the bodies may contain finely granular amorphous material, as well as coarser granular deposits, vesicles or curveted lamellae; acidic phosphatase activity has been demonstrated in MCB, that confirms they lysosomal origin. Almost identical

structures have been created *in vitro*, with gangliosides, cholesterol and phospholipids in molar ration of 1 to 3 to 1, incubated in an ionic medium. [125]
The composition of gangliosides from central nervous system of mammals is well known and characterized by GM1, GD1a, GD1b, GT1b, GQ1b and small amount of GM3 and GD3; these are present in proportions approximately preserved in different species and they correspond to different sialylation stage of the neutral chain Gal-GalNAc-Gal-Glc-Ceramide. Gangliosides are catabolized in the lysosomes by glycohydrolases that are exclusively exoglycosidases; the sequence of hydrolysis is:
GQ1b> GT1b> GDa1> GM1> GM2> GM3> LacCer> GlcCer> CerSph + Fatty Acids. [9]
Lysosomal diseases are most frequently classified according to the major storage compound [14] and, for differentiation of Gangliosidosis, biochemical analysis of the storage material with quantification of the different compounds can be a useful diagnostic and research tool. [126]
Particularly, GM1 gangliosidosis is characterized by the accumulation of GM1, GA1, GM2, GM3, GD1A, lyso-GM1, glucosylceramide, lactosylceramide, oligosaccharides, keratan sulphate. Instead, GM2 gangliosidosis, characterized by lack of hexosaminidase activity (necessary to pass from GM3 to GM2) is characterized by storage of GM2 and GD1aGalNac gangliosides. Moreover, differences between Tay-Sachs and Sandhoff diseases in storage gangliosides have been described: Tay-Sachs has increase in content of GA2 and lyso-GM2, while Sandhoff has increase globoside, oligosaccharides and lyso-GM2 portions. [14]
Biochemical analysis, particularly HPTLC analysis, showed striking increase of GM2 ganglioside in the brain of the affected wild boars, comparing to age- and sex-matched normal animals; this result allows to confirm the diagnosis of GM2 gangliosidosis with reduction of hexosaminidase activity.
Furthermore, was evident an increase in triesosil ceramide portion. This can happen when GM2 accumulates, because sialidase finds a high concentration

of substrate and is partially able to work by detaching the sialic acid, leaving the neutral triesosil ceramide.

Drastic reduction of principal components of myelin (sphingomyelin, sulphatide and cerebrosides) was evident in the organic phase. This biochemical value confirms what was evident with Luxol fast blue stain in histological samples and allows to confirm reduction of myelin content in wild boars' brains; as previously said, this is probably due to delayed or abnormal myelination (dysmyelinogenesis). [115]

# 8. Conclusions

Lysosomal storage diseases (LSDs) are rare genetic diseases, caused by deficient function of specific lysosomal enzymes. Gangliosides are major components of neuronal membranes; genetic defects in catabolism of these glycosphingolipids and their accumulation cause a specific disease, named Gangliosidosis. GM2 gangliosidosis is caused by β-hexosaminidase or GM2 activator protein deficiency, with GM2 ganglioside accumulation.

The aim of the project was to study morphological and biochemical features of GM2 gangliosidosis in 3 wild boars of the same brood.

These animals presented neurological signs at 6 months of age; due to the worsening conditions, they were euthanized at approximately one year of age. Gross examination of brains revealed reduction in consistency of the cerebral parenchyma; histological section of brain, cerebellum, spinal cord, peripheral ganglia and retinal revealed the presence of severely enlarge neurons, characterized by foamy vacuolated cytoplasm that multifocally peripheralized the nucleus and effaced the Nissl substance. Other findings were: spheroids, meganeurite, astrogliosis, microgliosis demyelination. Transmission Electron Microscopy (TEM) revealed the presence of elevate amount of cytoplasmic membranous bodies, defined as lysosomes filled by concentric or lamellar membranous material; these are characteristic of gangliosides storage. Biochemical analyses of the lipid content of the pathological brains, with high performance thin layer chromatography (HPTLC), revealed the presence of an elevate amount of GM2 ganglioside; this result allows to confirm the diagnosis of GM2 gangliosidosis with reduction of hexosaminidase activity. Furthermore, there was drastic reduction of principal components of myelin (sphingomyelin, sulphatide and cerebrosides), confirming the histological aspect.

To complete the study of this case of GM2 gangliosidosis in a family of wild boars, it would be necessary to determine if the mutation affected HEXA gene,

HEXB gene or GM2A gene, and which type of mutation is present. It would be also necessary to compare the mutation, in this family of wild boar, with those described in human medicine and in different animal species.

Since the human diseases are relatively rare, animal models are indispensable tools for further study of pathogenesis and for development of potential treatments. Spontaneous GM2 gangliosidosis has been described in numerous species; in swine, has been described only in 1978, in purebred Yorkshire swine (*Sus scrofa domestica*), but has never been described in several wild boars of the same brood (*Sus scrofa*).

Although, induced animal models have several limitations, due to differences with human pathology; this determines the necessity of spontaneous animal models, that have to be strictly similar to human LSD.

This form of GM2 gangliosidosis in a family of wild boars is very similar to human disease and can be a potential useful animal model.

# Bibliography


[1] V. Kumar, A. K. Abbas, N. Fausto and J. C. Aster, Robbins and Cotran Pathologic Basis of Disease, nona edizione ed., Elsevier Health Sciences, 2014.

[2] S. Santra and U. Ramaswami, "Lysosomal disorders," *Paediatrics and Child Health,* vol. 25, no. 3, pp. 123-132, march 2015.

[3] M. Xu, O. Motabar, M. Ferrer, J. J. Marugan, W. Zheng and E. A. Ottinger, "Disease models for the development of therapies for lysosomal storage diseases," *Annals of the New York Academy of Sciences,* 2016.

[4] T. Kolter and K. Sandhoff, "Sphingolipid metabolism diseases," *Biochimica et Biophysica Acta,* vol. 1758, p. 2057–2079, 2006.

[5] G. van Meer and A. I. P. M. de Kroon, "Lipid map of the mammalian cell," *Journal of Cell Science,* vol. 124, pp. 5-8, 2011.

[6] T. Kolter, "A view on sphingolipids and disease," *Chemistry and Physics of Lipids,* vol. 164, p. 590–606, 2011.

[7] E. Posse de Chaves and S. Sipione, "Sphingolipids and gangliosides of the nervous system in membrane function and dysfunction," *FEBS letters,* vol. 584, no. 9, pp. 1748-1759, 2010.

[8] F. Sabourdy and L. Astudillo, "Monogenic neurological disorders of sphingolipid metabolism," *Biochimica et Biophysica Acta,* vol. 1851, p. 1040–1051, 2015.

[9] T. Kolter and K. Sandhoff, " Lysosomal degradation of membrane lipids," *FEBS Letters,* vol. 584, p. 1700–1712, 2010.

[10] H. Schulze and K. Sandhoff, "Sphingolipids and lysosomal pathologies," *Biochimica et Biophysica Acta,* vol. 1841, p. 799–810, 2013.

[11] K. Sandhoff, "Neuronal sphingolipidoses: Membrane lipids and sphingolipid activator proteins regulate lysosomal sphingolipid catabolism," *Biochimie,* 2016.

[12] R. Jolly, "Lysosomal storage diseases of animals: an essay in comparative pathology," *Veterinary pathology,* vol. 34, pp. 527-548, 1997.

[13] S. D. Kingma, "Epidemiology and diagnosis of lysosomal storage disorders; challenges of screening," *Best Practice & Research Clinical Endocrinology & Metabolism,* vol. 29, p. 145e157, 2015.

[14] A. Ballabio and V. Gieselmann, "Lysosomal disorders: from storage to cellular damage," *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research,* vol. 1793, no. 4, pp. 684-696, 2009.

[15] D. Pelled, E. Lloyd-Evans, C. Riebeling., M. Jeyakumar and M. Platt, "Inhibition of calcium uptake via the sarco/endoplasmic reticulum Ca2+-ATPase in a mouse model of Sandhoff disease and prevention by treatment with N-butyldeoxynojirimycin," *Journal of Biological Chemistry,* vol. 278, no. 32, pp. 29496-29501, 2003.

[16] R. Buccoliero, J. Bodennec, V. Echten-Deckert, K. Sandhoff and A. H. Futerman, "Phospholipid synthesis is decreased in neuronal tissue in a mouse model of Sandhoff disease," *Journal of neurochemistry,* vol. 90, no. 1, pp. 80-88, 2001.

[17] C. Scriver, A. Beaudet, W. Sly and D. Valle, "β-galactosidase deficiency (β- galactosidosis): GM1 gangliosidosis and morquio B disease," in *The Metabolic and Molecular Bases of Inherited Disease*, New York, McGraw-Hill, 2001, p. 3775–3809.

[18] A. Pshezhetsky and M. Ashmarina, "Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology," *Prog. Nucleic Acid Res. Mol. Biol.,* vol. 69, pp. 81-114, 2001.

[19] G. Wu, Z. Lu, J. Wang, Y. Wang, X. Xie, M. Meyenhofer and R. Ledeen, "Enhanced susceptibility to kainate-induced seizures, neuronal

apoptosis, and death in mice lacking gangliotetraose gangliosides: protection with LIGA 20, a membrane-permeant analog of GM1," *J. Neurosci,* vol. 25, p. 11014–11022, 2005.

[20] J. Shield, J. Stone and C. Steward, "Bone marrow transplantation correcting beta-galactosidase activity does not influence neurological outcome in juvenile GM1-gangliosidosis," *J. Inherit. Metab. Dis,* vol. 28, p. 797–798, 2005.

[21] J. Matsuda, O. Suzuki, A. Oshima and Y. Yamamoto, " Chemical chaperone therapy for brain pathology in G(M1)-gangliosidosis," *Proc. Natl. Acad. Sci. U. S. A,* vol. 100, p. 15912–15917, 2003.

[22] J. Kasperzyk, A. d'Azzo, F. Platt, J. Alroy and T. Seyfried, " Substrate reduction reduces gangliosides in postnatal cerebrum-brainstem and cerebellum in GM1 gangliosidosis mice," *Substrate reduction reduces gangliosides in postnatal cerebrum-brainstem and cerebellum in GM1 gangliosidosis mice,* vol. 46, p. 744–751, 2005.

[23] T. Okumiya, H. Sakuraba, R. Kase and T. Sugiura, "Imbalanced substrate specificity of mutant beta-galactosidase in patients with Morquio B disease," *Mol. Genet. Metab.,* vol. 78, p. 51–58, 2003.

[24] R. Gravel, M. Kaback, R. Proia, K. Sandhoff, K. Suzuki and K. Suzuki, "The GM2 Gangliosidoses," in *The Metabolic and Molecular Bases of Inherited Disease*, vol. 3, New York, McGraw-Hill, 2001, p. 3827–3876.

[25] C. Lawson and D. Martin, "Animal models of GM2 gangliosidosis: utility and limitation," *The Application of Clinical Genetics,* vol. 6, no. 9, pp. 111-120, 2016.

[26] K. Suzuki and M. Vanier, "Biochemical molecular aspects of late-onset GM2-gangliosidosis: B1 variant as a prototype," *Dev. Neurosci.,* vol. 13, p. 288–294, 1991.

[27] E. Conzelmann and K. Sandhoff, "B variant of infantile GM2 gangliosidosis: deficiency of a factor necessary for stimulation of hexosami-

nidase A-catalyzed degradation of ganglioside GM2 glycolipid GA2," *Proc. Natl. Acad. Sci. U. S. A,* vol. 75, p. 3979–3983, 1978.

[28] R. Desnick and Y. Ioannou, "α-Galactosidase a deficiency fabry disease," in *The Metabolic and Molecular Bases of Inherited Disease*, 8 ed., New York, McGraw-Hill, 2001, p. 3733–3774.

[29] E. Beutler and G. Grabowski, "Gaucher Disease," in *he Metabolic and Molecular Bases of Inherited Disease*, 8 ed., New York, McGraw-Hill, 2001, p. 3635–3668.

[30] H. Zhao and G. Grabowski, "Gaucher disease: perspectives on a prototype lysosomal disease," *Cell Mol. Life Sci,* vol. 59, p. 694–707, 2002.

[31] M. Jmoudiak and A. Futerman, "Gaucher disease: pathological mechanisms and modern management," *Br. J. Haematol,* vol. 129, p. 178–188, 2005.

[32] K. v. Figura, V. Gieselmann and J. Jaeken, "Metachromatic leukodystrophy," in *The Metabolic and Molecular Bases of Inherited Disease*, 8 ed., New York, McGraw-Hill, 2001, p. 3695–3724.

[33] V. Gieselmann, S. Franken, D. Klein, J. Mansson and R. Sandhoff, "Metachromatic leukodystrophy: consequences of sulphatide accumulation," *Acta Paedriatr.,* vol. Suppl. 443, pp. 74-79, 2003.

[34] V. Gieselmann, "Metachromatic leukodystrophy: recent research developments," *J. Child Neurol,* vol. 18, pp. 591-594, 2003.

[35] D. Wenger, K. Suzuki, Y. Suzuki and K. Suzuki, "Galactosylceramide lipidosis: globoid cell leukodystrophy (Krabbe disease)," in *The Metabolic and Molecular Bases of Inherited Disease*, 8 ed., New York, McGraw-Hill, 2001, p. 3669–3694.

[36] M. Patterson, M. Vanier, K. Suzuki, J. Morris, E. Carstea, E. Neufeld, J. Blanchette-Mackie and P. Pentchev, "Niemann–Pick disease type C: a lipid trafficking disorder," in *The Metabolic and Molecular Bases of*

*Inherited Disease*, 8 ed., vol. 3, New York, McGraw-Hill, 2001, p. 3611–3633.

[37] E. Schuchman and R. Desnick, "Niemann–Pick disease types A B: acid sphingomyelinase deficiencies," in *The Metabolic and Molecular Bases of Inherited Disease*, 8 ed., New York, McGraw-Hill, 2001, p. 3589–3610.

[38] H. Moser, T. Linke, A. Fensom, T. Levade and K. Sandhoff, "Acid ceramidase deficiency: farber lipogranulomatosis," in *The Metabolic and Molecular Bases of Inherited Disease*, 8 ed., New York, McGraw-Hill, 2001, p. 3573–3588.

[39] M. Elleder, M. Jerabkova, A. Befekadu, M. Hrebicek, L. Berna, J. Ledvinova and H. Hulkova, "Prosaposin deficiency—A rarely diagnosed, rapidly progressing, neonatal neurovisceral lipid storage disease. Report of a further patient," *Neuropediatrics,* vol. 36, p. 171–180, 2005.

[40] R. Spiegel, G. Bach, V. Sury, G. Mengistu, B. Meidan, S. Shalev, Y. Shneor, H. Mandel and M. Zeigler, "A mutation in the saposin A coding region of the prosaposin gene in an infant presenting as Krabbe disease: first report of saposin A deficiency in humans," *Mol. Genet. Metab,* vol. 84, pp. 160-166, 2005.

[41] K. Kretz, G. Carson, S. Morimoto, Y. Kishimoto, A. Fluharty and J. O'Brien, "Characterization of a mutation in a family with saposin B deficiency: a glycosylation site defect," *Proc. Natl. Acad. Sci. U. S. A.,* vol. 87, pp. 2541-2544, 1990.

[42] H. Christomanou, A. Aignesberger and R. Linke, " Immunochemical characterization of two activator proteins stimulating enzymic sphingo-myelin degradation in vitro. Absence of one of them in a human Gaucher disease variant," *Biol. Chem. Hoppe-Seyler,* vol. 367, p. 879–890, 1986.

[43] J. Matsuda, M. Kido, K. Tadano-Aritomi, I. Ishizuka, K. Tominaga, K. Toida, E. Takeda, K. Suzuki and Y. Kuroda, "Mutation in saposin D domain of sphingolipid activator protein gene causes urinary system

defects and cerebellar Purkinje cell degeneration with accumulation of hydroxy fatty acid-containing ceramide in mouse," *Hum. Mol. Genet,* vol. 13, pp. 2709-2723, 2004.

[44] H. Zigdon, A. Meshcheriakova and A. Futerman, "From sheep to mice to cells: Tools for the study of the sphingolipidoses," *Biochimica et Biophysica Acta,* vol. 1841, pp. 1189-1199, 2014.

[45] K. Pierce, S. Kosanke, W. Bay and C. Bridges, "Animal model of human disease: GM2 gangliosidosis," *The American Journal of Pathology,* vol. 83, no. 2, pp. 419-422, 1976.

[46] S. Kosanke, K. Pierce and W. Bay, "Clinical and biochemical abnormalities in porcine GM2-gangliosidosis," *Vet Pathol,* vol. 15, no. 6, pp. 685-699, 1978.

[47] E. Karbe and B. Schiefer, "Familial amaurotic idiocy in male German shorthair pointers," *Pathol Vet,* vol. 4, p. 223–232, 1967.

[48] D. Read and D. Harrington, "Neuronal-visceral GM1 gangliosidosis in a dog with beta-galactosidase deficiency," *Science,* vol. 194, pp. 442-5, 1976.

[49] J. Alroy and U. Orgad, "Neurovisceral and skeletal GM1-gangliosidosis in dogs with beta-galactosidase deficiency," *Science,* vol. 229, pp. 470-472, 1985.

[50] G. Saunders and P. Wood, "GM1 gangliosidosis in Portuguese water dogs: pathologic and biochemical findings.," *Vet Pathol,* vol. 25, pp. 265-9, 1988.

[51] O. Yamato and K. Ochiai, "GM1 gangliosidosis in shiba dogs," *Vet Rec,* vol. 146, pp. 493-496, 2000.

[52] P. Whitfield and A. Johnson, "GM1-gangliosidosis in a cross-bred dog confirmed by detection of GM1-ganglioside using electrospray ionisation-tandem mass spectrometry," *Acta Neuropathologica,* vol. 100, pp. 409-414, 2000.

[53] G. Müller and S. Alldinger, "GM1-gangliosidosis in Alaskan huskies: clinical and pathologic findings.," *Vet Pathol,* vol. 38, pp. 281-90, 2001.

[54] Z. Wang and B. Zeng, "Isolation and characterization of the normal canine beta-galactosidase gene and its mutation in a dog model of GM1-gangliosidosis.," *J Inherit Metab Dis,* vol. 23, pp. 593-606, 2000.

[55] R. Kreutzer and T. Leeb, "A duplication in the canine beta-galactosidase gene GLB1 causes exon skipping and GM1-gangliosidosis in Alaskan huskies.," *Genetics,* vol. 170, pp. 1857-1861, 2005.

[56] B. Volk, E. Karbe and B. Schiefer, "Familiar amaurotic idiocy in male German shorthair pointers," *Vet Pathol,* vol. 4, p. 267, 1967.

[57] O. Yamato and N. Matsuki, "Sandhoff disease in a golden retriever dog.," *J Inherit Metab Dis,* vol. 25, pp. 319-20, 2002.

[58] S. Tamura and Y. Tamura, "GM2 Gangliosidosis Variant 0 (Sandhoff-Like Disease) in a Family of Toy Poodles," *J Vet Intern Med,* vol. 24, pp. 1013-1019, 2010.

[59] J. Cummings and P. Wood, "GM2 gangliosidosis in a Japanese spaniel," *Acta Neuropathol,* vol. 67, pp. 247-253, 1985.

[60] A. Freeman and S. Platt, "GM2 Gangliosidosis (B Variant) in Two Japanese Chins: Clinical, Magnetic Resonance Imaging and Pathological Characteristics," *J Vet Intern Med,* vol. 27, p. 771–776, 2013.

[61] M. Kohyama, A. Yabuki and Y. Kawasaki, "GM2 Gangliosidosis Variant 0 (Sandhoff Disease) in a Mixed-Breed Dog," *Journal of the American Animal Hospital Association,* vol. 52, no. 6, pp. 396-400, 2015.

[62] D. Sanders and R. Zeng, "GM2 gangliosidosis associated with a HEXA missense mutation in Japanese Chin dogs: A potential model for Tay Sachs disease," *Molecular Genetics and Metabolism,* vol. 108, pp. 70-75, 2013.

[63] M. Rahman and H. Chang, "A frameshift mutation in the canine HEXB gene in toy poodles with GM2 gangliosidosis variant 0 (Sandhoff disease)," *The Veterinary Journal,* vol. 194, pp. 412-416, 2012.

[64] H. Baker and J. Lindsey, "Neuronal GM1 gangliosidosis in a Siamese cat with beta-galactosidase deficiency.," *Science,* vol. 174, pp. 838-839, 1971.

[65] H. Baker and J. Lindsey, "Animal model: feline GM1 gangliosidosis," *Am J Pathol,* vol. 74, p. 649–652, 1974.

[66] C. Barker and W. Blakemore, "GM1 gangliosidosis (type 1) in a cat," *Biochemical Journal,* vol. 1, pp. 151-158, 1986.

[67] m. Uddin and m. Hossain, "Identification of Bangladeshi Domestic Cats with GM1 Gangliosidosis Caused by the c.1448G>C Mutation of the Feline GLB1 Gene: Case Study," *Journal of Veterinary Medical Science,* vol. 75, no. 3, pp. 395-397, 2013.

[68] H. Ueno and O. Yamato, "GM1 gangliosidosis in a Japanese domestic cat: a new variant identified in Hokkaido, Japan.," *J Vet Med Sci,* vol. 78, no. 1, pp. 91-5, 2016.

[69] R. De Maria and S. Divari, "Beta-galactosidase deficiency in a Korat cat: a new form of feline GM1-gangliosidosis," *Acta Neuropathol,* vol. 96, no. 3, pp. 307-314, 1998.

[70] D. Martin and B. Rigat, "Molecular consequences of the pathogenic mutation in feline GM1 gangliosidosis.," *Mol Genet Metab,* vol. 94, no. 2, pp. 212-221, 2008.

[71] L. Cork and J. Munnell, "GM2 ganglioside lysosomal storage disease in cats with beta-hexosaminidase deficiency.," *Science,* vol. 196, no. 4293, pp. 1014-1017, 1977.

[72] O. Yamato and S. Matsunaga, "GM2-gangliosidosis variant 0 (Sandhoff-like disease) in a family of Japanese domestic cats," *Vet Rec,* vol. 155, no. 23, pp. 739-744, 2004.

[73] D. Martin and N. Cox, "Mutation of the GM2 activator protein in a feline model of GM2 gangliosidosis.," *Acta Neuropathol,* vol. 110, no. 5, pp. 443-450, 2005.

[74] E. Neuwelt and W. Johnson, "Characterization of a new model of GM2-gangliosidosis (Sandhoff's disease) in Korat cats," *J Clin Invest,* vol. 76, no. 2, pp. 482-490, 1985.

[75] A. Bradbury and N. Morrison, "Neurodegenerative lysosomal storage disease in European Burmese cats with hexosaminidase beta-subunit deficiency.," *Mol Genet Metab,* vol. 97, no. 1, pp. 53-59, 2009.

[76] D. Martin and N. Cox, "Mutation of the GM2 activator protein in a feline model of GM2 gangliosidosis.," *Acta Neuropathol. ,* vol. 110, no. 5, pp. 443-50, 2005.

[77] L. Muldoon and E. Neuwelt, "Characterization of the molecular defect in a feline model for type II GM2-gangliosidosis (Sandhoff disease).," *Am J Pathol.,* vol. 144, no. 5, pp. 1109-1118, 1994.

[78] D. Martin and B. Krum, "An inversion of 25 base pairs causes feline GM2 gangliosidosis variant 0," *Experimental Neurology,* vol. 187, no. 1, pp. 30-37, 2004.

[79] Y. Kanae and D. Endoh, "Nonsense mutation of feline beta-hexosaminidase beta-subunit (HEXB) gene causing Sandhoff disease in a family of Japanese domestic cats.," *Res Vet Sci,* pp. 54-60, 2007.

[80] W. Donnelly, B. Sheahan and T. Rogers, "GM1-gangliosidosis in Friesian calves," *Journal of Pathology,* vol. 111, 1973.

[81] A. Ahern-Rindell and D. Prieur, "Inherited lysosomal storage disease associated with deficiencies of β-galactosidase and α-neuraminidase in sheep," *American Journal of Medical Genetics,* vol. 39, no. 1, pp. 39-56, 1988.

[82] B. Skelly, M. Jeffrey, F. RJ and B. Winchester, "A new form of ovine GM1-gangliosidosis.," *Acta Neuropathol,* vol. 89, no. 4, pp. 374-379, 1995.

[83] S. Ryder and M. Simmons, "A lysosomal storage disease of Romney sheep that resembles human type 3 GM1 gangliosidosis.," *Acta Neuropathol,* vol. 101, no. 3, pp. 225-228, 2001.

[84] B. F. Porter, B. C. Lewis, J. F. Edwards, J. Alroy, B. J. Zeng, P. A. Torres, K. N. Bretzlaff and E. H. Kolodny, "Pathology of GM2 Gangliosidosis in Jacob Sheep," *Veterinary Pathology,* vol. 48, no. 4, pp. 807-813, 2011.

[85] M. E. Wessels and J. P. Holmes, "GM2 Gangliosidosis in British Jacob Sheep," *J. Comp. Path.,* vol. 150, pp. 253-257, 2014.

[86] B. Zeng, P. Torres and T. Viner, "Spontaneous appearance of Tay–Sachs disease in an animal model," *Molecular Genetics and Metabolism,* vol. 95, pp. 59-65, 2008.

[87] J. Fox, Y.-T. Li and G. Dawson, "Naturally occurring GM2 gangliosidosis in two Muntjak deer with pathological and biochemical features of human classical Tay-Sachs disease (type B GM2 gangliosidosis)," *Acta Neuropathol,* vol. 97, pp. 57-62, 1999.

[88] T. Rickmeyer, S. Schoniger and P. A., "GM2 Gangliosidosis in an Adult Pet Rabbit," *J. Comp. Path,* vol. 148, pp. 243-247, 2013.

[89] S. Muthupalania, P. Torresb and B. Wang, "GM1-gangliosidosis in American black bears: Clinical, pathological, biochemical and molecular genetic characterization," *Molecular Genetics and Metabolism,* vol. 4, pp. 513-521, 2014.

[90] A. Bermudez, G. Johnson and M. Vanier, "Gangliosidosis in emus (Dromaius novaehollandiae).," *Avian Dis,* vol. 39, no. 2, pp. 292-303, 1995.

[91] A. R. Pinnapureddy, C. Stayner, J. McEwan, O. Baddeley, J. Forman and M. R. Eccles, "Large animal models of rare genetic disorders: sheep as phenotypically relevant models of human genetic disease," *Orphanet journal of rare diseases,* vol. 10, no. 1, p. 1, 2015.

[92] G. Maxie, Jubb, Kennedy & Palmer's Pathology of Domestic Animals, 6 ed., Elsevier Health Sciences, 2015.

[93] N. Cheville, "Ultrastructural Pathology: The Comparative Cellular Basis of Disease Autore," 2 ed., Wiley, 2009.

[94] C. Vogler, H. S. Rosenberg and Williams, "Electron microscopy in the diagnosis of lysosomal storage diseases," *American Journal of Medical Genetics,* vol. 28, no. s3, pp. 243-255, 1987.

[95] V. Papa, L. Tarantino and P. Preda, "The role of ultrastructural examination in storage diseases," *Ultrastructural pathology,* vol. 34, no. 5, pp. 243-251, 2010.

[96] E. Parkinson-Lawrence, T. Shandala and M. Prodoehl, "Lysosomal Storage Disease: Revealing Lysosomal Function and Physiology," *Physiology,* vol. 25, pp. 102-15, 2010.

[97] B. J. Skelly and &. R. J. Franklin, "Recognition and diagnosis of lysosomal storage diseases in the cat and dog," *Journal of veterinary internal medicine,* vol. 16, no. 2, pp. 133-141, 2002.

[98] O. Yamato and N. M. H. Satoh, "Laboratory diagnosis of canine GM2-gangliosidosis using blood and cerebrospinal fluid," *J Vet Diagn Invest,* vol. 16, pp. 39-44, 2004.

[99] M. M. Rahman, A. Yabuki, M. Kohyama, S. Mitani, K. Mizukami, M. M. Uddin and O. Yamato, "Real-time PCR genotyping assay for GM2 gangliosidosis variant 0 in Toy Poodles and the mutant allele frequency in Japan," *The Journal of Veterinary Medical Science,* vol. 76, no. 2, p. 295, 2014.

[100] S. Parikh, G. Bernard, R. J. Leventer, M. S. van der Knaap, J. van Hove, A. Pizzino and W. B. Rizzo, "A clinical approach to the diagnosis of patients with leukodystrophies and genetic leukoencephelopathies," *Molecular genetics and metabolism,* vol. 114, no. 4, pp. 501-515, 2015.

[101] J. Zimmerman, L. Karriker, A. Ramirez, K. Schwartz and G. Stevenson, in *Diseases of Swine*, 10 ed., Wiley-Blackwell, 2012.

[102] A. H. Fischer, K. A. Jacobson and Rose, "Hematoxylin and eosin staining of tissue and cell sections," *Cold Spring Harbor Protocols,* vol. 5, 2008.

[103] J. F. A. McManus, "Histological and histochemical uses of periodic acid," *Stain technology,* vol. 23, no. 3, pp. 99-108, 1948.

[104] Trump, Smuckler and Benditt, "A method for staining epoxy sections for light microscopy.," *Journal of ultrastructure research,* vol. 5, no. 4, pp. 343-348, 1961.

[105] W. Burns, "Thick Sections: Technique and Applications," in *Diagnostic Electron Microscopy*, New York, John Wiley & Sons, 1978.

[106] H. Klüver and E. Barrera, "A Method For The Combined Staining Of Cells And Fibers In The Nervous System," *Journal of Neuropathology & Experimental Neurology,* vol. 12, no. 4, pp. 400-403, 1953.

[107] H. Su-Ming, L. Raine and a. H. Fanger, "Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures," *Journal of Histochemistry & Cytochemistry,* vol. 29, no. 4, pp. 577-580, 1981.

[108] N. Cheville and J. Stasko, "Techniques in electron microscopy of animal tissue," *Veterinary Pathology,* vol. 51, no. 1, pp. 28-41, 2014.

[109] L. Graham and J. M. Orenstein, "Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research," *Nature protocols,* vol. 2, no. 10, pp. 2439-2450, 2007.

[110] F. Scandroglio, N. Loberto, M. Valsecchi, V. Chigorno, A. Prinetti and S. Sonnino, "hin layer chromatography of gangliosides," *Glycoconjugate journal,* vol. 26, no. 8, pp. 961-973, 2009.

[111] K. Suzuki, "Lysosomal diseases," in *Greenfield's Neuropathology*, 7 ed., London, Arnold, 2002, p. 654–64.

[112] S. Walkley, "Cellular Pathology of Lysosomal Storage Disorders," *Brain Pathology,* vol. 8, pp. 175-193, 1998.

[113] S. U. Walkley, H. J. Baker, M. C. Rattazzi, M. E. Haskins and J. Y. Wu, "Neuroaxonal dystrophy in neuronal storage disorders: evidence for major GABAergic neuron involvement," *Journal of the neurological sciences,* vol. 104, no. 1, pp. 1-8, 1991.

[114] M. Jeyakumar, R. Thomas, E. Elliot-Smith, D. A. Smith, A. C. Van Der Spoel and A. d'Azzo, "Central nervous system inflammation is a hallmark of pathogenesis in mouse models of GM1 and GM2 gangliosidosis," *Brain,* vol. 126, no. 4, pp. 974-987, 2003.

[115] D. Folkerth, "Abnormalities of developing white matter in lysosomal storage diseases," *Journal of Neuropathology & Experimental Neurology,* vol. 58, no. 9, pp. 887-902, 1999.

[116] M. Sofroniew and H. Vinters, "Astrocytes: biology and pathology," *Acta Neuropathol,* vol. 1119, pp. 7-35, 2010.

[117] F. Seehusen, E. Orlando, K. Wewetzer and W. Baumgärtner, "Vimentin-positive astrocytes in canine distemper: a target for canine distemper virus especially in chronic demyelinating lesions?," *Acta Neuropathol,* vol. 114, pp. 597-608, 2007.

[118] F. Chiu, W. T. Norton and K. Fields, "The Cytoskeleton of Primary Astrocytes in Culture Contains Actin, Glial Fibrillary Acidic Protein, and the Fibroblast-Type Filament Protein, Vimentin," *Journal of Neurochemistry,* vol. 37, no. 1, pp. 147-155, 1981.

[119] K. Rama Rao and T. Kielian, "Astrocytes And Lysosomal Storage Diseases," *Neuroscience 323,* vol. 23, p. 195–206, 2016.

[120] M. Hol and M. Pekny, "Glial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system," *Current opinion in cell biology,* vol. 32, pp. 121-130, 2015.

[121] Y. Wu, K. Mizugishi and M. Bektas, "Sphingosine kinase 1/S1P receptor signaling axis controls glial proliferation in mice with Sandhoff disease," *Hum Mol Genet,* vol. 17, no. 15, p. 2257–2264, 2008.

[122] N. Kawashima, D. Tsuji and T. Okuda, "Mechanism of abnormal growth in astrocytes derived from a mouse model of GM2 gangliosidosis," *J Neurochem,* vol. 111, no. 4, pp. 1031-1041, 2009.

[123] R. Wada, T. C.J. and P. R.L., "Microglial activation precedes acute neurodegeneration in Sandhoff disease and is suppressed by bone marrow transplantation," *Proc Natl Acad Sci U S A,* vol. 97, no. 20, p. 10954–9, 2000.

[124] Y. Wu and R. Proia, "Deletion of macrophage-inflammatory protein 1 alpha retards neurodegeneration in Sandhoff disease mice," *Proc Natl Acad Sci U S A,* vol. 101, no. 22, p. 8425–30, 2004.

[125] R. L. Friede, "Developmental Neuropathology," springer, 1989.

[126] T. Kobayashi, I. Goto, S. Okada, T. Orii, K. Ohno and T. Nakano, "Accumulation of lysosphingolipids in tissues from patients with GM1 and GM2 gangliosidoses," *Journal of neurochemistry,* vol. 59, no. 4, pp. 1452-1458, 1992.

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