Chapter 22
Lipidomics in Diagnosis of Lipidoses

C. Wolf and P.J. Quinn

Abstract A review is presented of the major clinical features of a number of glycolipidoses including Fabry, Gaucher, Tay-Sachs, metachromatic leukodystrophy as well as CeroidLipofucinosis and Sjogren-Larsson syndrome. The possibilities offered by lipidomics for diagnosis and follow-up after enzyme replacement therapy are presented from a practical perspective. The contribution of HPLC coupled with tandem mass spectrometry has considerably simplified the detection and assay of abnormal metabolites. Corresponding internal standards consisting of weighed mixtures of the stable-isotope labeled metabolites required to calibrate and quantitate lipid components of these orphan diseases standards have yet to become commercially available. A lipidomics approach has been found to compare favorably with DNA-sequence analysis for the rapid diagnosis of pre-birth syndromes resulting from these multiple gene defects. The method also seems to be suitable for screening applications in terms of a high throughput combined with a low rate of false diagnoses based on the wide differences in metabolite concentrations found in affected patients as compared with normal subjects. The practical advantages of handling samples for lipidomic diagnoses as compared to enzyme assay are presented for application to diagnosis during pregnancy.

Keywords Lipidomics · fabry · gaucher · Tay-Sachs · lipofucinosis · Sjogren-Larsson syndrome

22.1 Introduction

An interrogation of the Pubmed database for 2006–7 returns nearly 1000 articles and 132 reviews for "lipidoses". Of these the predominant subject is the glycolipidoses excluding sterol disorders (e.g., Niemann-Pick type C and

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deficits of cholesterol biosynthesis) and perturbations of energy-generating neutral glyceride metabolism which are not in the scope of this volume.

In the recent literature, Fabry disease, a deficiency of the lysosomal α-galactosidase A (EC 3.2.1.22) and subsequent accumulation in various tissues of globotriosylceramide (Gb3, Galα-(1→4)Galβ-(1→4)Glc-ceramide) and Gaucher disease, a deficit of glucocerebrosidase (GBA) are represented by 248 and 345 references, respectively. Other glycolipidosis frequently documented are Tay-Sachs disease, a deficiency of Hexosaminidase A causing the accumulation of gangliosides GM2 and metachromatic leukodystrophy, another lysosomal storage disorder caused by the deficiency in the sulfolipid degrading enzyme arylsulfatase A (ASA).

Of the deficits unrelated to the glycolipids which show an abundant "clinical" literature but less interest manifested by scientists in the field of lipidomics are ceroid lipofuscinosis (the deficiency in palmitoyl-protein thioesterase (PPT)) and the Sjogren-Larsson syndrome, a defect of ALDH3A2 gene which encodes fatty aldehyde dehydrogenase (FALDH). It is pertinent to question the reason for such differences between clinical and basic scientific interests reflected in literature citations.

The shift in the interest of many clinicians to the subject can easily be explained by the recent development of enzyme replacement therapy for some of these diseases. During the last decade this therapeutic strategy and the possibility of its use in prenatal diagnosis have excited interest in syndromes described more than 130 years ago. A current advance in therapy is the sophisticated and successful gene therapy developed recently for a frequent peroxisomal deficit of lipid metabolism, X linked adrenoleucodystrophy (Sevin et al., 2007). This method exploits an opportunity of gene transfection mediated by lentivirus. This is one of many treatments which have been critically reviewed by Beutler (Beutler, 2006) on the basis of their cost ($100–200 k/year) and efficacy. Other treatments at a lower cost, such as substrate depletion and chaperone therapy, are also examined in the review. As a result of these developments it is expected that basic studies focusing on deficits of lipid degradation and toxicity of accumulated by-products will be accelerated to reconcile the diverging views of pediatrians with health insurance.

We present in this Chapter an overview on these orphan diseases from the perspective of opportunities offered by developments in lipidomics. The severity of these conditions, which are usually progressively debilating, become manageable by therapies that considerably delay the appearance of the consequent irreversible damage.

The wide differences in the mutation rate inherent in these disorders govern the efforts of national associations of patients affected by lipidoses and that of enzyme replacement therapeutic industries. In turn, the clinical "everyday" practice triggers campaigns for the detection by genotyping combined with biochemical lipidomic methods which establish the screening efficiency in neonates. The history of cholesterol deficits such as the Smith-Lemli-Opitz

syndrome (SLOS) serves to illustrate how long it may take before the clinical and scientific aspects of disorders to be married. The clinical description of SLOS was first reported in 1964 (Smith et al., 1964). In the same year experimental teratologists established the activity of inhibitors of cholesterol synthesis for the mid-brain development and masculinization in rat embryos (Roux, 1964). Nevertheless, it has taken 30 years to recognize that blockade of cholesterol synthesis was the cause of SLOS (Tint, 1993). For this particular deficit a wide geographical distribution without any remarkably high incidence in small groups has most likely been responsible for a lack of an understanding of the underlying biochemical causes of the malformations. However most lipidoses presented in Table 22.1 have now received a clear biochemical explanation even if the detailed mechanisms of cell toxicity remain in many cases partially obscure.

Table 22.1 Summary of genetic defects, incidences and therapies of lipidoses

Syndrome	Enzyme deficit	Abnormal metabolite	Defective gene	Estimated incidence	Recent change in the status
Fabry	α-galactosidase A	Globotrihexosyl-ceramide (Gb3)	GLA (X-linked)	1:40000-1:55000	ERT
Gaucher	acid β-glucocere-brosidase	Glucoceramide	GBA or PSAP (saposin C, activor)	carrier up to ∼ 4% (Ashkenazi Jews)	ERT
Tay-Sachs	hexosaminidase A (α subunit)	Ganglioside GM2	HEXA	carrier ∼ 4% (in Ashkenazi Jews, French Canadians) to 0.4%	ERT
Metachromatic Leucody-strophy	arylsulfatase A	Cerebroside sulfate	ARSA	1:40000	LCMS2 screening of urinary sulfatides
Ceroid Lipofucinosis	palmitoyl-protein thioesterase (PPT 1)	granular osmiophilic deposits of acylated proteins	CNL1 (for PPT1) CNLx>10	1:12500	fluorimetric enzyme assay of PPT
Sjogren-Larson	fatty aldehyde dehydro-genase	Fatty alcohol	FALDH3	1:12000 north-east Sweden	GC assay of plasma aliphatic alcool

22.2 Fabry's Disease

The disorder results in an accumulation of globotriosylceramide (Gb3) in tissues due to deficiency of α-galactosidase activity.

22.2.1 Biochemical Features of Fabry's Disease

Gb3 which accumulates in cells of patients affected with Fabry's disease fulfils a number of regulatory processes. It has been known for many years that the glycosphingolipids act as a blood-group antigen, designated pk antigen (Naiki and Marcus, 1974). More recently,Gb3 has been shown to have a role as a receptor for shiga toxins (Lingwood, 1996), as a marker for the germinal centre stage of B-cell development (CD77) (Mangeney et al., 1993) and as an antigen associated with Burkett's lymphoma where it may be required for antigen presentation in malignant B-cells (George et al., 2001). The involvement of Gb3 in signaling *via* interferon-alpha pathways (Khine and Lingwood, 2000), CD19-mediated cell adhesion (Maloney and Lingwood, 1994) and apoptosis (Taga et al., 1997) has also been reported.

The role of Gb3 in its action as a plasma membrane receptor for internalization of Shiga-like toxins has been investigated in some detail. Binding of the toxin results in endocytosis and translocation via the endosomal system and *trans*-Golgi network in such a manner as to avoid the late endosomal compartment. Transport through the Golgi proceeds independently of coatormer protein-1 to the endoplasmic reticulum. There is evidence that Gb3 receptor functions are performed by the translocation of the glycosphingolipids into membrane microdomains on the cell surface. Thus treatment of cells in tissue culture with N-butyldeoxygalactonojirimycin, a specific inhibitor of ceramide glucosyl transferase, completely prevents uptake of toxin and induction of intracellular toxicity by denuding detergent-insoluble membrane fractions of Gb3 (Smith et al., 2006). These studies indicate that Gb3 function in cells is mediated by its sequestration in membrane rafts.

One of the attendant complications of Gb3 accumulation is the propensity towards vascular thrombosis (Utsumi et al., 1997). This is manifest in animal models such as the *Gla*-deficient mouse (Eitzman et al., 2003) but the connection is as yet unclear. This question has been examined by an analysis of tissue fibrin deposition in mice and thrombosis (Shen et al., 2006). The model confirmed a synergistic interaction between Gb3 and clotting factor V in tissue deposition suggesting an underlying explanation for susceptibility of stroke in Fabry's patients. The prothrombic state is associated with increased expression of the integrin CD11b on monocytes indicating leucocyte and endothelial activation (DeGraba et al., 2000). An elevation of blood levels of myeloperoxidase in Fabry's disease patients also contributes to risk factors in formation of atherosclerotic plaques generated, in part, by the production

of reactive oxygen species (Kaneski et al., 2006). Cerebral involvement in Fabry's disease is also believed to be due to vascular pathologies especially in young male patients (Moore et al., 2001, Moore et al., 2001). This is manifest as white matter lesions identified in cranial scans of both male and female patients (Fellgiebel et al., 2005)

22.2.2 Treatment and Monitoring

A concise presentation of this disorder has been given by European Fabry Outcome Survey group (Cybulla and Neumann, 2007): "Fabry's disease is a rare, X-chromosome linked recessive lysosomal storage disorder. In its course multiple organ damage occurs, e.g. in skin, nerves, kidneys and heart. If untreated the disease not only markedly impairs the quality of life but also shortens life expectancy. As it is a rare and not widely known disease with considerable variability of its symptoms it is often not or only belatedly diagnosed. Since 2001, enzyme replacement has become available as an option in the causal treatment." The opening lines clearly emphasize the critical role for clinicians to recognizing the condition as early as possible so as to trigger an appropriate diagnostic test. It also stresses that starting enzyme replacement therapy at an early stage of onset is imperative. However, the Survey Group indicates that usually the diagnosis is only made in adults after a long and deleterious period of time (Cybulla and Neumann, 2007) "Fabry Outcome Survey data bank for the documentation of the disease's clinical course shows on 262 patients (130 males, 132 females) a mean age 37.5 and 34 years, respectively, on entry in the FOS Typical symptoms". This is in contrast with the usual course where – acroparesthesias, joint pain, hypohidrosis, fever and angiokeratoma – have their onset in childhood (mean age nine years). The time interval is about 15 years between onset of the first symptoms and establishment of the diagnosis and the severity of the clinical picture correlates significantly with age ($p = 0.0001$). "Main causes of morbidity and death in Fabry's disease is involvement of the kidneys or heart, the one or other occurring in 75% of patients." What was a late diagnosis for a non-treatable deficit should now be changed into an early and widely available biochemical test since ERT is available.

A recent clinical trial of ERT (Banikazemi et al., 2007) shows that "Agalsidase-beta therapy slowed progression to the composite clinical outcome of renal, cardiac, and cerebrovascular complications and death compared with placebo in patients with advanced Fabry disease. Therapeutic intervention before irreversible organ damage may provide greater clinical benefit." The results of ERT are even better in children and emphasizes the importance of early detection prescribed by the pediatrician (Ries et al., 2006) "The boys showed a significant reduction in plasma globotriaosylceramide on treatment. Three patients (out of 24) with anhidrosis, as determined by quantitative sudomotor

axon reflex testing, developed sweating. Six of 11 patients could reduce or cease their use of antineuropathic analgesics." The results are appealing for a simple specific lipidomics method to detect an abnormal accumulation of globotriosylceramide.

In a pioneering study it was shown that infusion of α-galactosidase A reduced the tissue globotriaosylceramide storage in patients with Fabry disease (Schiffmann et al., 2000). Two methods were proposed in the trial with a potential diagnosis application: the assay of "total" Gb3 concentration by HPLC and a α -galactosidase A kinetics using the fluorogenic substrate 4-methylumbelliferyl- α -D-galactopyranoside. HPLC assay of perbenzoylated Gb3 (Ullman and McCluer, 1985; Ullman et al., 1985) can serve also to assay the distinct molecular species of glycolipids altogether. The procedure involves the conversion of gangliosides to their perbenzoyl derivatives, isolation of derivatives on a C18-reversed-phase cartridge, separation of the derivatives on a column (straight phase silica) maintained at an elevated temperature, and UV detection of the derivatives at 230 nm. The aspects of the procedure which contribute to its utility are a convenient isolation of derivatives and chromatographic conditions that provide the baseline resolution of derivatives. However the resolution of molecular species can also be obtained by LC coupled to tandem MS (Nelson et al., 2004; Roddy et al., 2005). "The accurate measurement of Gb3 in biological samples, i.e., plasma, is not trivial due to the inherent heterogeneity and amphiphilic nature of the Gb3 molecule. The structure of both the sphingoid base region (long-chain base), as well as the fatty-acyl chain region, can exhibit heterogeneity which increases the overall complexity of Gb3 measurements; the additional measurement complexity is attributed to the need to measure a large number of possible Gb3 isoforms". Indeed it has been suggested that monitoring of individual isoforms or specific isoform ratios in addition to the quantification of total Gb3 could yield an improved effectiveness of enzyme replacement therapy. A deuterated synthetic internal standard is now available for accurate quantitation of Gb3 with LC-MS (Mills et al., 2002). A non-invasive screening method for Fabry disease by measuring globotriaosylceramide in whole urine samples is now available using tandem MS. For instance the method allows a sensitive detection including cases in hemizygote girls (Kitagawa et al., 2005). Interference of the assay with the renal condition was noted in the reported results.

22.3 Gaucher's Disease

Gaucher's disease is an inborn error of sphingolipid metabolism caused by a deficiency of the lysosomal enzyme, acid β-glucosidase (EC 3.2.1.45) which is responsible for cleaving glucosylceramide (GlcCer) into glucose and

ceramide, a critical terminal step in many glycolipids catabolism (Brady et al., 1965, Brady et al., 1965).

22.3.1 Subcellular Changes

The subcellular catabolism of GlcCer has been examined using fluorescent derivatives of lactosylceramide. It was reported that lactosylceramide was targeted to late endosomes and lysosomes in fibroblasts and macrophage models of GD rather than to the Golgi in normal cells (Sillence et al., 2002). This effect was observed at levels of accumulated GlcCer well below that required to produce pathological signs of GD. Moreover, there was a concomitant increase in cholesterol content of the cells in these models inferring that cholesterol may be involved in miss-targeting because depletion of intracellular cholesterol restored the normal subcellular trafficking of lactosylceramide to the Golgi (Puri et al., 1999).

Globotriaosyl ceramide is required not only for Shiga toxin binding to cells, but also for its intracellular trafficking. Shiga toxin induces globotriaosyl ceramide recruitment to detergent-resistant membranes (Smith et al., 2006), and subsequent internalization of the lipid (Falguieres et al., 2006). The globotriaosyl ceramide pool at the plasma membrane is then replenished from internal stores. Whereas endocytosis is not affected in the recovery condition, retrograde transport of Shiga toxin to the Golgi apparatus (Falguieres et al., 2001) and the endoplasmic reticulum is strongly inhibited. This effect is specific, as cholera toxin trafficking on GM(1) and protein biosynthesis are not impaired. The differential behavior of both toxins is also paralleled by the selective loss of Shiga toxin association with detergent-resistant membranes in the recovery condition, and comparison of the molecular species composition of plasma membrane globotriaosyl ceramide indicates subtle changes in favor of unsaturated fatty acids.

More recently the effect of GlcCer accumulation on intracellular trafficking of other sphingolipids and phospholipids in the macrophage model of Gaucher's disease has been investigated (Hein et al., 2007). A 12-fold excess of GlcCer was induced in macrophages cultured in the presence of conduricol B-epoxide and the distribution of GlcCer in subcellular membranes was determined. It was found that initially GlcCer accumulates in lysosomes but as the content of the glycosphingolipids increases it becomes distributed relatively evenly throughout all the subcellular fractions. A similar pattern of accumulation of ceramide, di- and trihexosylceramides and phosphatidylglycerol was also observed in these cells suggesting that as the lysosomes become saturated with lipid the excess is shunted off to other subcellular compartments. The consequences of the excess glycosphingolipids and phosphatidylglycerol were said to interfere with biochemical pathways at these extralysosomal sites resulting in cell dysfunction and manifestation of the typical signs of Gaucher's disease.

22.3.2 Clinical Features

The deficiency of acid β-glucosidase manifests primarily in the macrophage. This is because macrophages acquire exogenously amounts of derived lipids from ingested senescent and apoptotic red and white blood cells. Therefore, the residual level of enzyme activity in the macrophage is insufficient to meet the needs of relatively large GlcCer turnover in these cells. Consequently GlcCer accumulates in the lysosomes of the mononuclear lineage throughout the reticulo-endothelial system including the liver, bone marrow, spleen and lung. Hematological involvement (thrombocytopenia and anemia), bone lesions and neurological impairment occur early in patients with certain severe genotypes (Koprivica et al., 2000). The prediction of disease progression in patients with Gaucher disease is difficult. The clinical manifestations of Gaucher disease are highly variable and, although certain genotypes are often associated with mild symptoms (N370S substitution) or severe (L444P), a simple correlation between genotype and phenotype does not exist because modifier genes play a modulatory role. However a relationship was found to exist among the 16:0-glucosylceramide/16:0-lactosylceramide ratio (glucosylceramide level is increased but lactosylceramide is decreased in plasma of more severely affected patients), LAMP-1 (lysosomal-associated membrane protein-1) and saposin C levels (non specific markers for lysosomal storage disorders) and the patient phenotype. The refinement of the genotype-phenotype correlation finding has major implications for the diagnosis, prediction of disease severity and monitoring of therapy in patients with Gaucher disease (Whitfield et al., 2002).

The size of the spleen has been shown to increase 25-fold in patients with Gaucher's disease. However it has been recently shown that a secondary overall sphingolipid accumulation in a macrophage model explains such an increase, GlcCer representing only 2% of the lipid mass. There is clear evidence from studies of the mouse model of Gaucher's disease, in which GlcCer accumulation is induced by inhibition of GlcCerase by conduritol B-epoxide, of perturbations in metabolism of phospholipids (Bodennec et al., 2002). The enlargement of cells with fibrillar and vacuolated cytoplasm, irregular nuclei and atypical subcellular membrane structures is believed to be associated with increased GlcCer content but it may not be entirely responsible for the observed phenotype. More recent studies of human macrophages treated with GlcCerase inhibitor showed additionally that the rate of synthesis of phosphatidylcholine was increased in these cells (Trajkovic-Bodennec et al., 2004). The accelerated rate of phosphatidylcholine synthesis is apparently due to an increase in activity of the key enzyme controlling the rate of synthesis, CTP:phosphocholine cytidylyltransferase (Kacher et al., 2007). The molecular mechanisms responsible for increased activity of CTP:phosphocholine transferase is presently unknown. Neither is it clear whether the increased synthesis of phosphatidylcholine resulting from GcCer accumulation contributes directly to the pathology of the disease.

22.3.3 Diagnosis and Treatment Monitoring

Biochemical identification and quantification of glucosyl- and lactosylceramide has widely benefited of LC tandem MS method. A general method using 1-phenyl-3-methyl-5-pyrazolone derivatives (Ramsay et al., 2005) of urinary oligosaccharides prior to analysis by electrospray ionization-tandem MS has been adapted to enable assay of large number of samples. PMP derivatization method was initially described by Honda (Honda et al., 1989). It can readily be extended to other oligosaccharidurias as a general method to monitor the levels of different oligosaccharide in patients receiving ERT. It has also potential for incorporation into a newborn screening program. The method semiquantifies urinary oligosaccharides from patients suffering from a variety of oligosaccharidurias including the Gaucher disease and GM1-GM2 gangliosidosis. The oligosaccharides are referenced against a single internal standard, methyl lactose, to produce ratios for a semiquantitative comparison with control samples. Elevations in specific urinary oligosaccharides were significantly indicative of lysosomal disease. The defective catabolic enzyme needs for specific assay a deuterated internal standard of the glycolipid to be incorporated in the sample before extraction.

22.4 Tay-Sachs Disease

Tay-Sachs disease is caused by the mutation of the alpha subunit of hexosaminidase A gene (HEXA). Deficitated hexosaminidases A and B produce 3 distinct clinical forms of ganglioside GM2 storage disease-Tay-Sachs disease, Sandhoff disease, and juvenile GM2-gangliosidosis. Hexosaminidase-A has a structure comprised of alpha-beta subunits and Tay-Sachs disease is the alpha-minus mutation, whereas Sandhoff disease is a beta-minus mutation (Beutler and Kuhl, 1975; Beutler et al., 1975). Subunit alpha is mapped to chromosome 15 (and beta to chromosome 5). Different levels of residual activities are correlated with the age of clinical onset: Tay-Sachs disease, 0.1% of normal hexosaminidase; late infantile, 0.5%; adult GM2-gangliosidosis, 2–4%; healthy persons with 'low hexosaminidase,' 11% and 20% (Conzelmann et al., 1983).

22.4.1 Genetic Distinction

Accumulation of GM2 is understood as a neurodegeneration that results in an excessive inflammatory reaction. Serial analysis of gene expression (SAGE) determined gene expression profiles in cerebral cortex from a Tay-Sachs patient, a Sandhoff disease patient and a pediatric control. Examination of

genes that showed altered expression in both patients revealed molecular details of the pathophysiology of the disorders relating to neuronal dysfunction and loss. A large fraction of the elevated genes in the patients could be attributed to activated macrophages/microglia and astrocytes, and included class II histocompatability antigens, the pro-inflammatory cytokine osteopontin, complement components, proteinases and inhibitors, galectins, osteonectin/SPARC, and prostaglandin D2 synthase (Myerowitz et al., 2002).

Enzyme screening of the serum remains an essential component of carrier screening in non-Jewish carriers because of uncommon mutations. DNA screening can be best used as an adjunct to enzyme testing to exclude known HEXA pseudo-deficiency alleles (Akerman et al., 1997). Tay-Sachs disease is approximately 100 times more common in infants of Ashkenazi Jewish ancestry (central-eastern Europe) than in non-Jewish infants (Rimoin et al., 1977). The most frequent (>80%) DNA lesion in Tay-Sachs disease in Ashkenazi Jews is a 4-bp insertion in exon 11 of the HEXA gene (Myerowitz and Costigan, 1988). These data strongly support the use of DNA testing alone as the most cost-effective and efficient approach to carrier screening for Tay-Sachs disease in individuals of confirmed Ashkenazi Jewish ancestry (Bach, et al., 2001).

22.4.2 Enzyme Screening and Lipidomics

Methods for the assay of hexosaminidase A and total hexosaminidase activities in dried blood spots on filter paper offer considerable advantages for screening (Chamoles et al., 2002). The deficient activity of the lysosomal enzymes hexosaminidase A and total hexosaminidase (hexosaminidase A plus B) are usually measured in plasma or extracts of leukocytes. To tubes containing a 3-mm-diameter blood spot, elution liquid and substrate solution were added. After incubation at 37°C, the amount of hydrolyzed product was compared with a calibrator to allow the quantification of enzyme activity. The method was proven reliable even after storage for up to 38 months at room temperature. For total hexosaminidase, the substrate was 4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (neutral derivative) and for hexosaminidase A, 4-methyl-umbelliferyl-β-D-*N*-acetyl-glucopyranoside-6-sulfate. For ERT therapeutic evaluation of GM2 gangliosidoses ELISA using anti-GM2 ganglioside antibodies are now used for intracellular quantification of GM2 (Tsuji et al., 2007). Another method for profiling gangliosides extracted from animal tissues is using ESI-MS/MS with high through-put potential (Tsui et al., 2005). This system utilizes specific detection of a precursor ion (m/z 290), a derivative of *N*-acetylneuraminic acid comprised in gangliosides. The method includes the enrichment of gangliosides in the aqueous phase from total cellular lipid extracts which eliminates the damping effect of phospholipids and permits direct precursor scan.

22.5 Metachromatic Leucodystrophy

Metachromatic leukodystrophy is another lysosomal storage disorder caused by a deficiency of arylsulfatase A which leads to the accumulation of 3-O-sulfogalactosylceramide. The defect results in severe demyelination. The disease often takes a presentation of an under- and mis-diagnosed psychiatric affection long before neurological symptoms appear and MRI displays the anatomical lesions. MRI reveals a diffuse demyelination, bilateral and often symmetrical, initially limited to the periventricular areas.

22.5.1 Genetic Prevelance

In a recent multicenter study two mutations were found the most prevalent amongst the European population: "c.459 + 1G>A and p.P426L, in 384 unrelated European patients presenting with different types of metachromatic leukodystrophy were found. In total, c.459 + 1G>A was found 194 times among the 768 investigated ARSA alleles (25%), whereas P426L was identified 143 times (18.6%). Thus, these two mutations accounted for 43.8% of investigated MLD alleles." As a function of age and geographical distribution the 2 mutations were found very different (Lugowska et al., 2005) "Mutation c.459 + 1G>A was most frequent in late-infantile MLD patients (40%), while P426L was most frequent in adults (42.5%). Mutation c.459 + 1G>A is more frequent in countries situated at the western edges of Europe, i.e., in Great Britain and Portugal, and also in Belgium, Switzerland, and Italy, which is visible as a strand ranging from North to South, and additionally in Czech and Slovak Republics. Mutation P426L is most prevalent in countries assembled in a cluster containing the Netherlands, Germany, and Austria."

22.5.2 Biochemical Characteristics

Studies of the animal model have established a link between biosynthesis of sulfogalactosylceramides, accumulation and demyelination. The sulfatide storage pattern in ASA-deficient [ASA(−/−)] mice is comparable to humans, but the mice do not mimic the myelin pathology. It was assumed that increasing sulfatide storage in this animal model might provoke demyelination. Transgenic ASA(−/−) mice overexpressing the sulfatide-synthesizing enzyme galactose-3-O-sulfotransferase-1 [tg/ASA(−/−)] were prepared. Indeed, these tg/ASA(−/−) mice displayed a significant increase in sulfatide storage in brain and peripheral nerves and older than 1 year mice developed severe neurological symptoms related to demyelination (Eckhardt et al., 2007; Ramakrishnan et al., 2007). The accretion of sulfatides is directly correlated with the neurological phenotype. In generated transgenic ASA-deficient [ASA(−/−)] mice overexpressing the sulfatide synthesizing enzymes UDP-galactose:ceramide galactosyltransferase

(CGT) and cerebroside sulfotransferase (CST) neuronal lipid storage was provoked. CGT-transgenic ASA(−/−) [CGT/ASA(−/−)] mice showed an accumulation of C18:0 fatty acid-containing sulfogalactosylceramide in the brain. Histochemicaly, an increase in sulfolipid storage could be detected in central and peripheral neurons of both CGT/ASA(−/−) and CST/ASA(−/−) mice compared with ASA(−/−) mice. CGT/ASA(−/−) mice developed severe neuromotor coordination deficits and weakness of hindlimbs and forelimbs.

22.5.3 Clinical Signs

The biochemical abnormalities as a function of the presentation have been recently scrutinized by Bauman's group (Colsch et al., 2007) "During adolescence and/or adulthood, there are 2 clinical presentations. It may be that of a degenerative disease of the central nervous system with mainly spastic manifestations or a spino-cerebellar ataxia, or that of a psychosis. As several lines of evidence indicate that the psychotic form of MLD could be a model of psychosis, we decided to do a pluridisciplinary study on 11 psycho-cognitive cases involving mental and psychiatric testing, in comparison with 5 adult motor cases. However a biochemical study with enzyme assays and quantitative mass spectrometry of urinary sulfatides, so as to determine whether there were biochemical particularities related to the psychotic forms does not show any difference." These biochemical data have yet to be reconciled with previous observations where a phenotype-genotype correlation was described (Rauschka et al., 2006) "P426L homozygotes principally presented with progressive gait disturbance caused by spastic paraparesis or cerebellar ataxia; mental disturbance was absent or insignificant at the onset of disease but became more apparent as the disease evolved. In contrast, compound heterozygotes for I179S presented with schizophrenia-like behavioral abnormalities, social dysfunction, and mental decline, but motor deficits were scarce. Reduced peripheral nerve conduction velocities and less residual arylsulfatase A activity were present in P426L homozygotes *vs* I179S".

22.5.4 Lipidomics

Sulfatides (3-sulfogalactosylceramides) were initially detected by mass spectrometry using FAB ionization but spectrum showed a number of fragmentations which complicates considerably the method (Ohashi and Nagai, 1991). More recently structural characterization of sulfatides by collisional-activated dissociation (CAD) was described in quadrupole ion-trap tandem mass spectrometric methods with electrospray ionization. With the method [M – H]- ions of sulfatides yield abundant structurally informative ions that permit unequivocal assignments of the long-chain base and fatty acid constituent including the location of double bond (Hsu and Turk, 2004). The major sulfatide molecular species are quantified similarly in the 2 clinical forms (motor and

psycho-cognitive adult forms) with the following fatty acids and sphingoid bases: C22:1/d18:1 and /or C22:0/d18:2 (m/z 862.5), C22:0 (OH)/d18:1 (m/z 878.5), C24:0/d18:1 and / or C24:0/C23:1(OH)/d18:2 (m/z 890.3), C24:0 (OH)/d18:1(m/z 906.5) (Colsch et al., 2007). Because the diagnosis may be complicated in cases of arylsulfatase A pseudodeficiency and sphingolipid activator protein deficiency, this measurement of sulfatide in the urinary sediment of affected individuals by a rapid, sensitive, and specific mass spectrometric method has been long wanted (Whitfield et al., 2001). Urinary sulfatides are now commonly detected using electrospray ionization-tandem mass spectrometry by means of the precursor ion scan 97. Levels are considerably increased to X20–30 folds as compared to controls which allows the rapid screening of a large number of samples.

22.6 Ceroid Lipofuscinoses

The condition is caused by deficiencies of palmitoyl protein thioesterase 1 (PPT1) (or tripeptidyl peptidase 1 (TPP1) and possibly other enzymes resulting in the same clinical presentation of NCL (for *N*eural *C*eroid *L*ipofuscinose)). PPT1 cleaves long-chain fatty acids from S-acylated proteins within the lysosome (Lu et al., 1996). How the loss of this activity causes the death of central nervous system neurons is not known.

22.6.1 Clinical Aspects

A strong interest for this group of disorders was recently raised after a clinical trial (Steiner et al., 2007) has been conducted to evaluate the safety and preliminary efficacy (phase 1) of human central nervous stem cells (HuCNS-SC) implanted into the cortex and lateral ventricles of patients with advanced neuronal ceroid lipofuscinoses. It is known that the accumulation of undigested substrates leads to the formation of neuronal storage bodies that are associated with the clinical symptoms. With the same clinical presentation, TPP1 gene mutation is related to the deficiency of tripeptidyl peptidase 1. The corresponding CLN2 gene product is synthesized as an inactive proenzyme that is autocatalytically converted to an active serine protease (Lin et al., 2001). The deficit of this lysosomal protease causes neuronal ceroid lipofuscinoses, a group of inherited, neurodegenerative, lysosomal-storage disorders characterized by intracellular accumulation of autofluorescent ceroid lipofuscin storage material in neurons and other cells. The patients experience progressive cognitive and motor deterioration, blindness, seizures (as early as 2–3 yrs) and early death.

The two subtypes of NCLs are due to deficiencies either in the palmitoyl protein thioesterase 1 (PPT1) protein or in the tripeptidyl peptidase 1 (TPP1) protein. The trial aforementioned by a cell therapy was comprised of neural stem/progenitor cells which constitutively synthesize and secrete both the PPT1 and TPP1 enzymes. In culture, these secreted enzymes are internalized by

fibroblasts from patients with the PPT1 or TTP1 deficiency. Transplantation of HuCNS-SCs into PPT1 knockout immunodeficient mice leads to global engraftment, provides PPT1 enzyme, reduces storage material, neuroprotects host neurons, and extends survival of host mice.

22.6.2 Enzymology

The recent development of simple, fluorogenic enzyme assay using 4-methylumbelliferyl-6-thiopalmitoyl-ß-glucoside (MUTG) for infantile and late infantile neuronal ceroid lipofuscinosis has greatly facilitated the diagnostic process for these diseases (Young et al., 2001). In leucocytes and fibroblasts from infantile patients profound deficiencies of palmitoyl-protein thioesterase 1 (PPT1) are found, the residual activity being $< 5\%$ of mean control. The feasibility of a reliable prenatal enzyme analysis was tested successfully using the fluorogenic substrate. In fibroblasts from late infantile lipofuscinoses patients a similar syndrome, the deficiency of tripeptidyl-peptidase I activity (TPP-I) was frequently found. More than 30 mutations have been reported altogether in PPT1 and TPP1 genes, rendering the molecular genetic analysis impractical as a primary means of diagnosis. Electron microscopy of characteristic cellular inclusions remains an important diagnostic method, but it is also tedious and not readily available. A simple assay for the determination of tripeptidyl peptidase and palmitoyl protein thioesterase activities in dried blood spots is now available (Lukacs, et al., 2003). The clinical presentation of ceroid lipofuscinoses is especially difficult to correlate with a single biochemical mechanism. Indeed after reviewing 319 patients with NCL, the authors (Wisniewski et al., 2001) found that 64 (20%) did not fit into gene defects for *CLN1* and *CLN2* which encode lysosomal palmitoyl protein thioesterase and tripeptidyl peptidase 1, respectively. Eight NCL forms are now considered which result from 100 different mutations.

22.7 Sjogren-Larsson Syndrome

The syndrome is caused by a particular mutation in the gene encoding fatty aldehyde dehydrogenase (gene FALDH3A2). About 1.3% of the population of northern Sweden is heterozygous for the defective gene.

22.7.1 Clinical Aspects

Sjogren and Larsson described in 1957 patients with spastic diplegia or tetraplegia, low grade oligophrenia, and ictyosiform erythrodermia developing during infancy. Many patients have also characteristically retinal glistering

spots. The ophtalmological abnormalities are often severe in the syndrome. Detailed information is given on this complication in (Aslam and Sheth, 2007; Romanes, 1968). About half the affected children have an early pigmentary degeneration of the retina (juvenile macular dystrophy) (Willemsen et al., 2000). Most of the patients never walk and about half the patients have seizures.

22.7.2 Genetic and Enzymic Characteristics

The evidence that Sjögren-Larsson syndrome is genetically homogeneous was critically analyzed by two references (Pigg et al., 1999; Rogers et al., 1995). It is assumed that Swedish soldiers bivouacking in Germany during the 30-year war in the 17th century could have introduced the Sjogren-Larsson gene into the German population. A missense mutation in the FALDH gene was maped on chromosome 17 in Sjögren-Larsson syndrome patients originating from the northern part of Sweden. Fatty alcohol:NAD^+ oxidoreductase, the enzyme catalyzing the oxidation of hexadecanol or octadecanol to the corresponding fatty acid is deficient in fibroblasts (mean activity at 13% of that in normal fibroblasts). Fibroblasts from heterozygotes show intermediate levels of activity (Rizzo et al., 1987; Rizzo et al., 1989).

Fatty alcohol:NAD^+ oxidoreductase is a complex enzyme which consists of two separate proteins that sequentially catalyze the oxidation of fatty alcohol to fatty aldehyde and to fatty acid that is to say a fatty alcohol dehydrogenase and a fatty aldehyde dehydrogenase (FALDH) activity. Sjögren-Larsson cells were selectively deficient in the FALDH component and had normal activity of fatty alcohol dehydrogenase. Intact fibroblast oxidized octadecanol to fatty acid at $<10\%$ of the normal rate but oxidized octadecanal normally confirming that FALDH is specifically affected (Rizzo and Craft, 1991).

Sjögren-Larsson can be diagnosed prenatally using enzymatic methods (Rizzo et al., 1994). Fatty alcohols in cultured cells and plasma were analyzed as acetate derivatives using capillary column gas chromatography. By this method, cultured skin fibroblasts from Sjögren-Larsson patients were found to have 7- and 8-fold elevations in the mean content of hexadecanol (16:0-OH) and octadecanol (18:0-OH), respectively. Most importantly, the mean plasma 16:0-OH and 18:0-OH concentrations in Sjögren-Larsson patients ($n = 11$) were 9- and 22-fold higher than in normal controls (about 10 ng/ml), respectively. In fibroblasts, most of the fatty alcohol (59%) that accumulated was free rather than esterified alcohol, whereas free alcohol accounted for 23% of the total alcohol in normal cells indicating that elevations in free fatty alcohols provide a sensitive marker for Sjögren-Larsson syndrome. Furthermore, dietary or cell media sources may contribute to the tissue or cell content of specific fatty alcohols.

22.8 Lipidomics Applied to Diagnosis

The characteristical lipid metabolites (glycolipids or fatty alcohols) which accumulate in lipidoses are reviewed above. None represents a particular difficulty to authentify or assay quantitatively as regard to the multiple lipids which have already been successfully studied by the lipidomics. The method for usual lipids is commonly understood as a process comprising extraction, solvent partition and a critical HPLC-tandem MS step to ascertain the metabolite structure and allow a (semi)-quantitative assay. For glycolipids and for highly polar gangliosides (TSD) or cerebroside sulfate (MLD), specifically, the partition between alcoholic water layer and chloroformic lower layer (Bligh and Dyer, 1959; Folch, et al., 1957) cannot be applied as it is for most of the lipids with a moderate or no hydrophilicity at neutral or acidic pH. With the perspective of clinical application a variety of methods were challenged for gangliosides from red blood cells (Wang and Gustafson, 1995). Results show that ganglioside extraction is unfavourably affected by the addition of the solvents as a mixture and by the use of less polar solvents and by a lower total solvent-to-sample ratio. The distribution of gangliosides could be uneven in an apparently monophasic extraction solvent mixture. The uneven distribution occurred during and also after the extraction (in filtration and centrifugation). In the recommended method using 19 volumes of methanol/chloroform (2:1) solvent in a one-step extraction, the above disadvantages in ganglioside extraction and quantification are kept under control. This method appears simple and it gives a high recovery of gangliosides. Pre-analytical steps in the perspective of HPLC-tandem MS serves to separate the metabolite of interest from compounds which may suppress ion formation in the ESI source. Usually suppression is caused by abundant ions with a charge similar with the metabolite.

In the case of acidic glycolipids the relative proton affinity of chemicals can shift the balance for negative ionisation in favor of co-eluted compounds. Pre-analytical separation under acidic conditions serves also to reduce as much as possible the "dispersion" in the MS spectrum of the metabolite into multiple m/z representing the various adducts of counterions Na^+, K^+, NH_4^+, organic amines$^+$,... which improves sensitivity of the test. Sulfatides are lost during the partition between the hexane and the methanol/water phase. The analysis of sulfatides involves the isolation of the glycosphingolipid fraction and the subsequent separation of sulfatides from neutral lipids by chromatography on DEAE-sephadex or DEAE-cellulose column (the variety of methods are referenced in the website CyberLipid (http://www.cyberlipid.org/).

The contribution of lipidomics for the diagnosis of any severe defect related to a metabolic inherited abnormality should be judged taking into account 2 criteria; the 1srt is the delay required for the laboratory response given to the obstetrician after sampling (amniotic fluid or chorionic villosity) and the 2nd criterion is the difficulty for an alternative molecular DNA diagnostic when no specific but multiple mutations have to be searched for along a multi-kb gene.

Based on everyday practice lipidomics methods are well able to provide the ascertained diagnostic without false positive or negative on very long periods of time (>15 yrs). Answers are usually given within few days and delay can be furthermore reduced after the sample transfer from the diagnostic center is optimized and the electronically certified results are directly mailed to the clinician in charge with the patient. Lipids metabolites as compared to most other diagnostics requiring enzyme assay do not require any particular precautions in terms of sample transfer. Postal mail with no cooling or freezing of the biological sample can be achieved with the advantages of a modest cost for transportation and the critical possibility to collect samples for a regional or national lipidomics unit appropriately trained. The reduction of delay for a positive ascertained diagnostic leaves a possibility for a medical abortion if required. If the severe defect is evoked by sound-examination at the term of 10–12 weeks of gestation mifepristone abortifacient may be applied instead of the surgical uterine revision. By difference DNA analysis of multi-kb genes (such as CNL or TSD in non-Ahskenazi population) and multiple polymorphisms may imply time-consuming PCR amplification and tedious analysis of the sequence.

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