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# Arylsulfatase A: Genetic Epidemiology and Structure / Function Studies

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# ARYLSULFATASE A: GENETIC EPIDEMIOLOGY AND STRUCTURE/ FUNCTION STUDIES

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## PRECEITOS LEGAIS

Esclarece-se serem da nossa responsabilidade a execução das experiências que estiveram na origem dos resultados apresentados neste trabalho, assim como a sua interpretação, discussão e redacção.

Nesta tese foram apresentados resultados contidos nos artigos, publicados ou em vias de publicação, seguidamente discriminados:

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**ABSTRACT**

Arylsulfatase A (ARSA) is the lysosomal enzyme implicated in most cases of Metachromatic Leukodystrophy (MLD), a rare but severe lysosomal storage disorder belonging to the group of sphingolipidosis. A relatively frequent condition among the general population is denominated Arylsulfatase A Pseudodeficiency (ARSA-PD), and is characterised by a low activity of this enzyme, not associated with the MLD clinical phenotype.

In this work, the genetic epidemiology of both these ARSA deficiencies have been studied by the molecular characterisation of Portuguese MLD patients and by the determination of the frequency of the ARSA-PD associated alleles in the general population. Among the patients characterised, we identified eight different mutations, four of them being novel. For mutation IVS2+1G>A, one of the most frequent null mutations associated with late infantile MLD, we found the highest frequency reported in Europe (60%). Among the general Portuguese population, approximately 18% of the individuals are carriers of one of the three ARSA-PD associated alleles. This is the highest value reported in a European population and demonstrates the importance of this condition in Portugal.

Analysis of the haplotype associated with ARSA-PD alleles and with the MLD causal mutations was also done, in order to contribute to the elucidation of the origin, spreading and actual distribution of these alterations among the populations, and in this way also contributing to the identification of risk groups. The possible existence of common ancestors for several MLD causal mutations, including the null mutation IVS2+1G>A, is suggested by our results. In the case of the ARSA-PD associated alleles, only one of them has a conserved haplotype, thus suggesting a unique origin.

Expression studies were performed that proved the causality of three novel missense mutations (F219V, C300F, P425T). With the aim of contributing to the elucidation of the genotype-phenotype correlations in MLD, the biochemical characterisation of these mutations was also done. All of them were found to cause a reduction in ARSA activity, two of them having almost null activity (F219V, C300F). All the mutated enzymes were shown to be unstable, to have reduced specific activity and to be partially retained in the endoplasmic reticulum. Mutations P425T and C300F were both found to interfere with ARSA octamerization and this may be the cause of an increased accessibility of P425T-ARSA and C300F-ARSA to the lysosomal proteases, thus explaining their low stability.

The existence of a general genotype-phenotype correlation in MLD seems to be corroborated by our results, in spite of the finding of some inter and intrafamilial heterogeneity among the adult patients.

Although the individuals with ARSA-PD do not develop the clinical symptoms characteristic of MLD, the possibility of its contribution to the pathogenesis of other pathologies has been speculated for long. To try to contribute to the clarification of this question, the frequency of the ARSA-PD associated alleles was also determined in a group of Portuguese schizophrenic patients. Our results seem to indicate a higher susceptibility of ARSA-PD individuals to develop schizophrenia.

## RESUMO

A Leucodistrofia Metacromática (MLD) é uma doença lisossomal de sobrecarga rara, mas grave que se inclui no grupo das esfingolipidoses e que na maioria dos casos é devida à deficiência na enzima lisossomal Arilsulfatase A (ARSA). Na população em geral observa-se com relativa frequência uma condição denominada Pseudodeficiência em Arylsulfatase A (ARSA-PD), que é caracterizada por uma baixa actividade desta enzima, mas não está associada ao fenótipo clinico da MLD.

Neste trabalho estudou-se a epidemiologia genética destas duas deficiências em ARSA através da caracterização molecular de doentes portugueses com MLD e da determinação da frequência dos alelos associados à ARSA-PD na população portuguesa em geral. O estudo dos doentes portugueses com MLD permitiu a identificação de oito mutações diferentes, quatro das quais identificadas neste trabalho pela primeira vez. Relativamente à mutação IVS2+1G>A, uma das mutações nulas mais frequentemente associada com a forma clinica infantil tardia da MLD foi encontrada a frequência mais elevada até à data descrita numa população europeia de doentes com MLD (60%). Na população portuguesa em geral, verificou-se ainda que aproximadamente 18% dos indivíduos são portadores de um dos três alelos associados à ARSA-PD. Este é o valor mais elevado já descrito numa população europeia e demonstra a importância desta condição em Portugal.

A análise do haplótipo associado aos alelos responsáveis pela ARSA-PD e aos alelos causais da MLD também foi efectuada, numa tentativa de contribuir para a elucidação da origem, dispersão e distribuição actual destas alterações em diferentes populações, desta forma contribuindo também para a identificação de grupos de risco. Os resultados obtidos sugerem a existência de ancestrais comuns para várias mutações

causais da MLD, incluindo a mutação IVS2+1G>A, cuja elevada frequência em Portugal já foi referida. Relativamente aos alelos associados à ARSA-PD, os resultados obtidos sugerem uma origem única para apenas um deles, cujo haplótipo é conservado.

Para confirmar a causalidade de três mutações missense descritas de novo (F219V, C300F, P425T) foram efectuados estudos de expressão. Com o objectivo de contribuir para a elucidação da correlação genótipo-fenótipo na MLD, foi também efectuada a caracterização bioquímica destas mutações. As três mutações estudadas causam a redução da actividade da ARSA; para duas delas (F219V, C300F) a actividade observada é praticamente nula. Foi também observada instabilidade, actividade específica reduzida e retenção parcial no retículo endoplasmático, para as três enzimas mutadas estudadas. Os estudos efectuados permitiram ainda observar que as mutações P425T e C300F interferem com a octamerização da ARSA. Esta menor capacidade de octamerização poderá contribuir para o aumento da acessibilidade das proteínas mutadas às proteases lisossomais, explicando desta forma a sua baixa estabilidade. Apesar de ter sido observada alguma heterogeneidade inter e intrafamiliar, particularmente entre os doentes adultos, os resultados obtidos neste trabalho parecem também corroborar a existência de uma correlação genótipo-fenótipo geral na MLD.

Apesar dos indivíduos com ARSA-PD não desenvolverem os sintomas clínicos característicos da MLD, a possibilidade da contribuição desta condição para a patogénese de outras doenças constitui desde há muito uma hipótese que continua em estudo. Para tentar contribuir para a clarificação desta questão, a frequência dos alelos associados à ARSA-PD também foi determinada num grupo de doentes portugueses com esquizofrenia. Os resultados obtidos sugerem uma maior susceptibilidade dos indivíduos portadores da ARSA-PD para desenvolver esquizofrenia.

**RESUME**

La Leucodystrophie Métachromatique (MLD) est une maladie lysosomale de surcharge rare, mais néanmoins grave qui est inclut dans le groupe des sphingolipidoses et qui, dans la plupart des cas, est due à une déficiente activité de l' enzyme du lysosome Arylsulfatase A (ARSA). Dans la population en général, on observe, avec relative fréquence, une condition dénommée Pseudodéficience en Arylsulfatase A (ARSA-PD), qui est caractérisée par une diminuition importante de l'activité de cette enzyme, mais dont la diminuition d'activité n' est pas associée au phénotype clinique de la MLD.

Dans ce travail, on a étudié l'épidémiologie génétique de ces deux types de déficiences en ARSA à travers la caractérisation moléculaire des malades portugais avec MLD e la détermination de la fréquence des allèles associés à l' ARSA-PD dans la population portugaise en général. L' étude des malades portugais avec MLD a permis l'identification de huit mutations différentes, dont quatre ont été décrites pour la première fois dans ce travail. En ce qui concerne la mutation IVS2+1G>A, une des mutations nulles plus fréquement associée à la forme clinique infantile tardive de la MLD, on a découvert que la fréquence de cette mutation (60%) est la plus élevée parmi les populations européennes de malades avec MLD. On a vérifié que dans la population portugaise à peux prés 18% des individus sont hétérozygotes pour un des trois allèles associés à l'ARSA-PD. Cette fréquence est la plus élevé des fréquences décrites dans les pays européens ce qui pose des problémes particuliéres dans l'étude de la MLD au Portugal.

L'analyse de l' haplotype, associé aux allèles responsables de l'ARSA-PD et aux allèles pathologiques de la MLD, a aussi été réalisé dans le but d' élucider l'origine, la

dispersion et la distribution actuelle de ces altérations dans des differents populations, ce qui peu permettre l'identification des groupes de risque. Les résultats obtenus suggèrent l' existence d' ancêtres communs pour diverses mutations pathologiques de la MLD, ce qui est aussi valable pour la mutation IVS2+1G>A, dont on a déjà référée la fréquence élevée au Portugal. Relativement aux allèles associés à l'ARSA-PD, seulement pour un de ces allèles, dont l'haplotype a été conservé, les résultats obtenus suggèrent une origine unique.

Pour confirmer la causalité des trois nouvelles mutations missenses (F219V, C300F, P425T), on a réalisé des études d' expression. Dans le but de déterminer l' existence d' une corrélation génotype-phénotype dans la MLD, on a aussi réalisé la caractérisation biochimique de ces mutations. Les trois mutations étudiées provoquent une réduction de l'activité de l'ARSA, et deux de ces mutations (F219V, C300F) originent une activité presque nulle. Differents characteristiques des trois enzymes mutées ont été étudiées ce qui a permit d'observer l' instabilité, la diminuition de l' activité spécifique et la rétention partielle au niveau du réticulum endoplasmique. Les études réalisées ont permis aussi d' observer que les mutations P425T et C300F interfèrent avec l' octamérization de l'ARSA. Cette diminuition de leur capacité d' octamérization peut contribuer pour une meilleure accessibilité des protéines mutées aux protéases lysosomiques, ce qui explique leur faible stabilité. Malgré le fait d'une certaine hétérogénéité inter et intra familial, particulièrement dans les cas des malades adultes, les résultats obtenus dans ce travail semblent aussi corroborer l'existence d'une corrélation génotype-phénotype dans la MLD.

Même si les individus ARSA-PD ne dévelloppent pas les symptômes cliniques caractéristiques de la MLD, la possibilité de la contribution de cette condition pour la

pathogenèse d'autres maladies constitue depuis longtemps une hypothèse à confirmer. Afin de clarifier cette question, la fréquence des allèles associés à l' ARSA-PD a aussi été déterminée dans un groupe de malades portugais schizophréniques. Les résultats obtenus suggèrent que les individus hétérozygotes pour un des alléles ARSA-PD ont une plus grande susceptibilité pour le dévellopment de la schizophrénie.

## ABBREVIATIONS

| | |
|---|---|
| ARSA | Arylsulfatase A |
| ARSA-PD | Arylsulfatase A Pseudodeficiency |
| ARSB | Arylsulfatase B |
| BHK | Baby Hamster Kidney |
| BMT | Bone Marrow Transplantation |
| CD-MPR | Cation-Dependent mannose-6-Phosphate receptor |
| CGT | UDP-galactose:ceramide galactosyltransferase |
| CI-MPR | Cation-Independent mannose-6-Phosphate receptor |
| CNS | Central Nervous System |
| CSF | Cerebro Spinal Fluid |
| CT | Computed Tomography |
| ER | Endoplasmic Reticulum |
| FGE | Formylglycine-generating enzyme |
| FGly | Formylglycine (2-amino-3-oxopropionic acid) |
| LSD | Lysosomal Storage Disorders |
| Man-6-P | Mannose-6-Phosphate |
| MSD | Multiple Sulfatase Deficiency |
| MLD | Metachromatic Leukodystrophy |
| MRI | Magnetic Resonance Imaging |
| PAPS | 3'-Phosphoadenosine-5'-Phosphosulfate |

| | |
|---|---|
| PNS | Peripheral Nervous System |
| SAP | Saposin |
| SRP | Signal-Recognition Particle |

# GENERAL INTRODUCTION

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## 1.1. Lysosome and lysosomal proteins

### 1.1.1. Endocytic and biosynthetic pathways to the lysosome

Lysosomes are the major intracellular site for final degradation of a large variety of macromolecules that get there through the endocytic pathway, through the biosynthetic pathway or by fusion with phagosomes. They are part of a very complex endosomal network, which also includes endosomes and carrier vesicles, and whose importance is more and more recognised. The early endosomes are located in the peripheral areas of the cell and receive ligands and solutes from the extracellular space, through vesicles derived from the plasma membrane. Once inside the early endosomes, most ligands detach from their receptors due to the low endosomal pH, some are targeted to the lysosomes, others are recycled back to the cell surface together with the receptors (for review see Lloyd, 1996; Smythe, 1996).

"Volume rich regions" of the early endosomes are transferred to the late endosomes. These are heterogeneous structures, with many internal membranes, that can be observed in the perinuclear region of the cell, and are the organelles to which the lysosomal enzymes are delivered (for review see Lloyd, 1996; Smythe, 1996).

Lysosomal enzymes are delivered to the late endosome in clathrin-coated vesicles derived from the *trans*-Golgi network. These vesicles recycle back to the Golgi, together with the enzyme-receptors that are induced to release their cargo at low pH. Some lysosomal enzymes can also be delivered to the plasma membrane, from where they can be reinternalised by receptor-mediated endocytosis. In this case they enter the

endocytic pathway at the early endosome level (for review see Lloyd, 1996; Smythe, 1996).

### 1.1.2. Normal pathways of biosynthesis and transport of soluble lysosomal proteins

The pathway of lysosomal enzymes' biosynthesis involves transport through multiple compartments before they reach the lysosome: they are first directed to the endoplasmic reticulum (ER) where they are subjected to several post-translational modifications; they then transit through the Golgi, where they are further processed; they exit the Golgi via clathrin-coated vesicles and are delivered to the late endosomes. Upon reaching the lysosomes many of them are proteolytically processed (for review see Kornfeld *et al.*, 1989).

The initial steps in the biosynthesis of soluble lysosomal proteins are the same as for secretory proteins and many membrane proteins. They have a signal sequence, which consists of a stretch of 15-30 mainly hydrophobic amino acids at the N-terminus, and that interacts with a signal-recognition particle (SRP). They are then targeted to the ER through the interaction of the SRP with the SRP-recognition docking protein and afterwards translocated into the ER's lumen, where the signal peptide is cleaved even before their synthesis being completed (von Figura *et al.*, 1986).

Still in the ER, selected asparagine residues in the sequence Asn-X-Thr/Ser (where X means any amino acid except proline and asparagine) are core glycosylated with a pre-formed high mannose type oligosaccharide ($Glc_3Man_9GlcNac_2$), donated by a dolichol pyrophosphate. This oligosaccharide is further processed in several reactions catalysed by Glucosidase I and II and alpha-mannosidase, which remove the glucose residues and one of the mannose residues. The first reaction specific to the synthesis of

soluble lysosomal enzymes is the acquisition of the Mannose-6-phosphate (Man-6-P) recognition marker that occurs in the Golgi, and that is catalysed by the sequential action of two enzymes. In a first reaction, a phosphotransferase (UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase) transfers N-acetylglucosamine 1-phosphate from UDP-N-acetylglucosamine to mannose residues of high mannose-type oligosaccharide side chains of lysosomal enzymes, originating N-acetylglucosamine-1-phospho-6-mannose residues. Then, the "uncovering" enzyme (N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase) removes the terminal N-acetylglucosamine residue, exposing the Man-6-P residues. Both enzymes are multisubunit proteins. The phosphotransferase is responsible for the specificity of the acquisition of Man-6-P markers by the lysosomal enzymes. This selective binding is affected by the oligosaccharide moieties of the lysosomal enzymes but the critical factor is the presence of particular conformation-dependent protein domains in these enzymes. Several studies suggest that these recognition domains are surface patches with critical lysine residues correctly spaced relative to each other and to the oligosaccharide moiety (for review see Kornfeld *et al.*, 1989; Braulke, 1996; Kornfeld *et al.*, 2001).

The deficiency in the phosphotransferase is the biochemical defect of the genetic disorder I-Cell disease. To date no patients have been found that lack the "uncovering" enzyme (Kornfeld *et al.*, 2001).

### 1.1.3. Mannose-6-phosphate receptors

The Man-6-P markers are responsible for the specific, high affinity binding of acid hydrolases to one of the two mannose-6-phosphate receptors, which occurs in the

*trans*-Golgi network or in the plasma membrane. These receptors mediate the targeting of lysosomal enzymes to lysosomes by both the biosynthetic and endocytic pathways. Two distinct Man-6-P receptors have been isolated and characterised, a cation-independent mannose 6-phosphate receptor (CI-MPR) and a cation-dependent mannose 6-phosphate receptor (CD-MPR), they are related integral membrane glycoproteins with apparent molecular weights of 215 KDa and 46 KDa, respectively. Both receptors have three structural domains: an N-terminal extracytoplasmic domain, a single transmembrane domain and a C-terminal cytoplasmic domain. The extracytoplasmic domain of the large receptor consists of 15 repeating elements, each homologous to the extracellular domain of the small receptor. This seems to indicate that the large receptor arose from multiple duplications of a single ancestral gene. In the biosynthetic pathway, each receptor seems to be able to functionally compensate for the loss of the other, in spite of the better efficiency of the CI-MPR. In the endocytic pathway, the CD-MPR functions very inefficiently (for review see Kornfeld *et al.*, 1989; Braulke, 1996; Kornfeld *et al.*, 2001).

## 1.2. Lysosomal Storage Disorders

The deficient catabolic activity of lysosomal hydrolases or the deficiency of lysosomal transporter proteins, results in the blockade of the lysosomal digestive process with the subsequent accumulation, within the lysosome, of undigested substrates that can be observed as abnormal intracellular inclusions characteristic of lysosomal storage disorders (LSD), (Suzuki *et al.*, 2002).

More than forty distinct genetic lysosomal disorders have been identified and the enzyme deficiency underlying the majority of these disorders has been elucidated.

Clinically, they consist of an heterogeneous group and usually they are classified into different subgroups, according to the nature of the undigested accumulated substrates. Although this classification is to some extent arbitrary, two major groups are sphingolipidoses and mucoplysaccharidoses, which are respectively characterised by the accumulation of sphingolipids and glycosaminoglycans due to their defective degradation. Glycoprotein storage disorders constitute a third group of LSD in which the degradation of carbohydrate chains of glycoproteins are affected resulting in the accumulation of oligosaccharides and glycoproteins. Neuronal ceroid lipofuscinosis refers to another clinically heterogeneous group of LSD characterised by the accumulation of ceroid lipofuscin inclusions. Apart from these groups there are still a miscellaneous of LSD characterised by the accumulation of several distinct substrates (for review see Suzuki *et al.*, 2002).

## 1.3. Metachromatic Leukodystrophy

### 1.3.1. General definition and incidence

Metachromatic Leukodystrophy (MLD) is a lysosomal storage disorder belonging to the group of sphingolipidoses. It is inherited as an autosomal recessive trait, and is characterised by the intralysosomal accumulation of sulfatide (galactosyl-3-sulfate ceramide) in different tissues and by its elevated excretion in urine, due to the deficient activity of the lysosomal enzyme Arylsulfatase A (E.C.3.1.6.8, ARSA) or to the activator protein Saposin B (SAP B) deficiency (for review see von Figura *et al.*, 2001). Figure 1 depicts the catabolism of sphingolipids with the indication of the enzymes involved and the related lysosomal disorders. The step blocked by ARSA activity deficiency is emphasized.

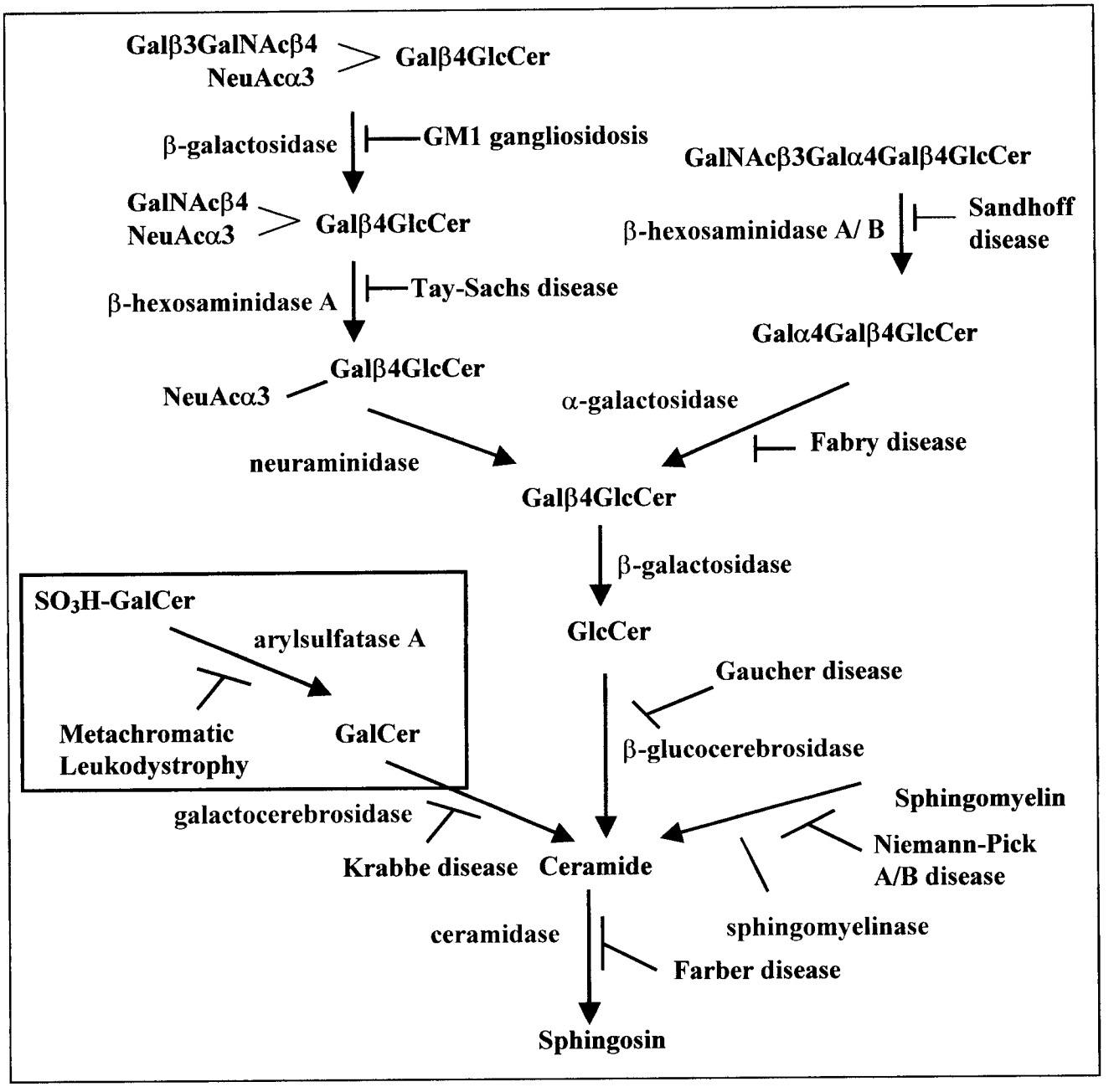



**Figure 1 – Catabolism of major sphingolipids: enzymes involved and enzyme related diseases.** Abbreviations: Cer – ceramide; Gal – galactose; GalCer – galactosylceramide; GalNAc – N-acetylgalactosamine; Glc – glucose; GlcCer – glucosylceramide; NeuAc – N-acetylneuramic acid (sialic acid), (Suzuki *et al.*, 2002).

The overall incidence of MLD is not accurately determined. Several studies, in different populations, indicate an incidence between 1:40000 and 1:170000 for the late infantile MLD due to ARSA deficiency (Gustavson *et al.*, 1971; Guibaud *et al.*, 1973; Farrell *et al.*, 1981; Heim *et al.*, 1997). In Australia the prevalence of MLD was determined to be 1: 92000 (Meikle *et al.*, 1999). Higher incidences of MLD have been reported for several isolated ethnic groups: 1:75 in the Habbanite Jews living in Israel (Zlotogora *et al.*, 1980), 1:8000 in the Christian Arabs living in Israel (Heinisch *et al.*, 1995), 1:2500 in the Eskimos and 1:6400 in the Navajo Indians (Pastor-Soler *et al.*, 1994; Pastor-Soler *et al.*, 1995). Regarding MLD due to Sap B deficiency, only nine cases have been described up to now (Sandhoff *et al.*, 2001).

### 1.3.2. Clinical forms and pathophysiology

According to the age of onset, there are three distinct MLD clinical forms associated with the deficiency of ARSA activity: a late infantile, a juvenile and an adult form. Late infantile patients usually show normal development during their first year of live, the first symptoms occurring before the 4$^{th}$ year. Progressively, these patients start to loose the ability to walk, stand and later even to sit. After the motor disturbances, impairment of language and mental function also develops. In the later stage of the disease, the child reaches an unresponsive vegetative state, and dies usually within 2 to 4 years after onset of symptoms. The clinical features of the juvenile form of MLD resemble those of the late infantile form, but the age of onset varies from 4 to the late teens. Emotional and behavioural disturbances are frequently observed. The progression of the illness is slower and it may extend over a decade. The adult form of MLD has been reported at various ages beyond puberty, and patients can present in a very

different way. The initial manifestations may be signs of emotional lability, delusions, psychotic behaviour and dementia. Motor disturbances may only be observed in the final stages of the disease, which may follow several decades after the onset of the emotional and mental disturbances (for review see Graljaard, 1980).

Several complementary examinations can be important for the clinical diagnosis of MLD. Brain Computed Tomography (CT), Magnetic Resonance Imaging (MRI), measurements of nerve conduction velocity and evoked potential studies are of the major importance. An abnormal low density of the cerebral white matter can be observed at very early stages of the disease and neurophysiological studies reveal decreased motor nerve conduction velocity and diminished amplitude of sensory nerve action potentials, indicating the presence of peripheral neuropathy. The brainstem auditory evoked responses and the visual evoked responses can also be abnormal. Late infantile patients have increased urinary sulfatide excretion and increased CSF protein and sulfatide content. Juvenile and adult patients usually also have elevated urinary sulfatide excretion, but CSF protein can present normal values. Normal CSF sulfatide have also been observed even in late stages of juvenile and adult MLD. The examination of the gallbladder can also be important because alterations can be observed even before the occurrence of the first neurological symptoms (for review see von Figura *et al.*, 2001).

The morphologic changes characteristic of MLD are demyelination and deposits of metachromatic granules in the central and peripheral nervous system and in the visceral organs. These granules have a diameter of 15 to 20 μm, and are composed mainly of sulfatide (39%), cholesterol and phosphatides (Suzuki *et al.*, 1967).

In the central nervous system (CNS) metachromatic granules are visible within macrophages, oligodendrocytes and certain groups of neurons (Takashima *et al.*, 1981). The amount of CNS myelin is reduced and its "cavitation or spongy degeneration" can be observed. A diminished number of oligodendrocytes and a reactive gliosis in the demyelinated areas are also observed (von Figura *et al.*, 2001).

In the peripheral nervous system (PNS), the metachromatic granules are present in Schwann cells and in endoneurial macrophages (Martin *et al.*, 1982). There is a reduced myelin sheath thickness for all fibers, but the ones with the greater axon diameter are the most affected (Alves *et al.*, 1986; Bardosi *et al.*, 1987).

The progressive intralysosomal accumulation of sulfatide is not uniform in all tissues and organs and can lead to a loss of function or contribute to the fatal outcome of the disease, depending on the tissue. Several organs accumulate sulfatide: the kidneys (LeRoy *et al.*, 1973; Eto *et al.*, 1982), gallbladder (Tesluk *et al.*, 1989; Malde *et al.*, 1997), liver parenchymal cells and epithelial cells of the intrahepatic bile ducts (Resibois *et al.*, 1971), islets of Langerhans (Hagberg *et al.*, 1962), anterior pituitary (Wolfe *et al.*, 1964), adrenal cortex (Wolfe *et al.*, 1964) and the sweat glands (Goebel *et al.*, 1989). In adult patients, metachromatic material accumulation was also reported in the testes, this is probably not sulfatide but seminolipid. Lactosylsulfatide levels are also increased, especially in the kidney (Scott *et al.*, 1993).

Besides the increase in the amount of sulfatides, a marked decrease in the level of other myelin lipids like galactocerebroside, cholesterol and sphingomyelin can also be observed (Norton *et al.*, 1982; Stallberg-Stenhagen *et al.*, 1965). The galactocerebroside:sulfatide ratio can be decreased from 3 (approximate value for normal white matter) to 1 (Svennerholm *et al.*, 1963; Harzer *et al.*, 1987) and the

amount of lysosulfatide, the deacylated form of sulfatide, can be increased 50-100 times (Toda *et al.*, 1989; Toda *et al.*, 1990).

The demyelination occurring in MLD seems to be secondary to the sulfatide induced metabolic failure of the Schwann cells (PNS) and oligodendrocytes (CNS), and at least three different mechanisms have been suggested to explain the myelin breakdown that is observed in MLD patients. It can result from the defective resorption of sulfatides in the innermost part of the myelin sheath, it can be due to an altered structure leading to its instability or it can result from the accumulation of lysosulfatide, a highly cytotoxic compound whose accumulation is described in different tissues of MLD patients. The demyelination of long tracts can explain the decreased nerve conduction velocity, the decreased synaptic transmission and the increase in the latency period of evoked potentials that are observed in MLD patients. The intellectual impairment can result from the damage of the neurons and from a scrambling of information due to the slow nerve conduction velocity (for review see von Figura *et al.*, 2001).

## 1.4. Structural similarities of sulfatases and biochemical diagnosis of the sulfatases' deficiencies

### 1.4.1. The sulfatases' family

Thirteen different sulfatases participate in the hydrolysis of complex sulfate ester substrates in the vertebrates (Parenti *et al.*, 1997). Eight of these sulfatases are involved in the removal of sulfate from glycosaminoglycans, glycopeptides and glycolipids, and are active in the lysosome. The other ones participate in the hydrolysis of

hydroxysteroids and are found in microsomes, endoplasmic reticulum and Golgi network (for review see Hopwood *et al.*, 2001).

The identification of at least eight human genetic diseases caused by the deficiency of individual sulfatase activities revealed the crucial importance of each of these enzymes in the catabolism of specific sulfated substrates. The deficiency in the lysosomal sulfatases that act on glycosaminoglycans originates five different types of mucopolysaccharidoses (Neufeld *et al.*, 2001) whereas the deficiency in arylsulfatase A, the lysosomal sulfatase involved in the hydrolysis of a glycolipid originates the glycosphingolipidosis MLD (von Figura *et al.*, 2001). X-linked ichthyosis and chondrodysplasia punctata are caused by deficiencies in the non-lysosomal sulfatases arylsulfatase C and arylsulfatase E, respectively (Franco *et al.*, 1995; Ballabio *et al.*, 2001). There is also a rare disorder, Multiple Sulfatase Deficiency (MSD), in which the activity of all sulfatases is deficient and the patients have clinical features characteristic of MLD, mucopolysaccharidosis, X-linked ichthyosis and chondrodysplasia punctata. This was the first indication that sulfatases have a common structural feature essential for their catalytic activity (Hopwood *et al.*, 2001).

The members of this sulfatases' family are highly conserved from bacteria to man, with a very high degree of homology between their amino acid sequence (Franco *et al.*, 1995; Parenti *et al.*, 1997; Knaust *et al.*, 1998; von Figura *et al.*, 1998) and their three-dimensional structure (Bond *et al.*, 1997; Lukatela *et al.*, 1998; Waldow *et al.*, 1999). The N-terminal third of these proteins presents the highest homology, including a cysteine that is converted to a Cα-formylglycine amino acid derivative (FGly), and that was found in sulfatases of prokaryotic (Dierks *et al.*, 1998; Miech *et al.*, 1998), lower eukaryotic (Selmer *et al.*, 1996) and human origin (Schmidt *et al.*, 1995). This

FGly residue (2-amino-3-oxopropionic acid) results, in all eukaryotic sulfatases, from the oxidation of a cysteine residue (Schmidt *et al.*, 1995), and probably occurs at a late cotranslational or early posttranslational step of protein tanslocation (Dierks *et al.*, 1997; Fey *et al.*, 2001). The finding that this posttranslational modification is required for the activity of all sulfatases and that it is defective in the patients with MSD, suggested that the molecular basis of this disease resides in a gene or genes codifying the cysteine to formylglycine conversion machinery (Schmidt *et al.*, 1995). Although it was known for some time that the FGly-modifying machinery consists of soluble components of the ER (Fey *et al.*, 2001), only very recently the formylglycine-generating enzyme (FGE) was identified (Cosma *et al.*, 2003; Dierks *et al.*, 2003). FGE is codified by a nine exons gene (SUMF1) that spans approximately 106 Kb in chromosome 3p26. The cDNA encoding human FGE predicts a protein with 374 amino acids and a single Asn glycosylation site. FGE resides in the endoplasmic reticulum and seems to be both essential and limiting for the synthesis of catalytically active sulfatases. Another protein, codified by a gene designated SUMF2, was also identified as having some capacity to activate sulfatases, but is significantly less active than SUMF1 and its importance in MSD remains to be elucidated (Cosma *et al.*, 2003; Dierks *et al.*, 2003).

### 1.4.2. Biochemical diagnosis of MLD

In spite of its high specificity for their natural substrates "in vivo", some sulfatases cleave a wide range of synthetic substrates "in vitro", which can raise some difficulties in the biochemical diagnosis of some sulfatases enzymatic deficiencies.

The biochemical diagnosis of MLD is usually done by ARSA activity determination in peripheral blood leukocytes or fibroblasts, using artificial substrates as the p-nitrocatechol sulfate or 4-methylumbelliferyl sulfate. However, these substrates are also degraded by other sulfatases and thus require the use of assay conditions that selectively diminish their interference, specially the one from the co-localizing lysosomal sulfatase Arylsulfatase B (ARSB). Different assays have been developed for determination of ARSA activity based on the inhibition of ARSA and ARSB by $Ag^{+}$ and high concentrations of NaCl, respectively (Baum *et al.*, 1959; Christomanou *et al.*, 1977). The differential tolerance of arylsulfatases toward low temperatures can also be used to efficiently distinguish ARSA and ARSB activities (Lee-Vaupel *et al.*, 1987).

Another possibility to increase the specificity of the assays is the physical separation of the different arylsulfatases that can be accomplished by ion exchange chromatography (Humbel, 1976) or electrophoretic techniques (Chang *et al.*, 1984; Alves *et al.*, 1986; Li *et al.*, 1992). The use of radioactively labelled sulfatide, the natural substrate of ARSA, allows the specific determination of its activity, but it is a very expensive, time consuming and technically difficult method (Leinekugel *et al.*, 1992).

Besides the existing problems in the determination of ARSA activity, two different conditions cause important problems in the biochemical diagnosis of MLD: the Arylsulfatase A pseudodeficiency (ARSA-PD), a condition not associated with the clinical symptoms of MLD and that will be discussed later in this chapter, and MLD due to Saposin B deficiency. In the case of ARSA-PD, low ARSA activity levels are measured "in vitro" using artificial substrates, in the case of MLD due to Saposin B deficiency, normal ARSA levels are determined with artificial substrates. In both cases

MLD can be excluded or proved by a sulfatide-loading assay in cultured fibroblasts (Kihara *et al.*, 1980; Fluharty *et al.*, 1978) and by a urine sulfatide determination (Molzer *et al.*, 1992).

## 1.5. Natural substrates of ARSA

### 1.5.1. Biological importance of sulfatide and other natural substrates of ARSA

Sulfatides (galactosylceramide 3-sulfate) are the main sphingosine-containing sulfated glycolipids whose lysosomal accumulation is characteristic of MLD. They occur in higher amounts in central and peripheral nervous system and in the kidney but can also be observed in other tissues, particularly those with a rich excretory epithelium (Krivan *et al.*, 1989; Natomi *et al.*, 1990; Takamatsu *et al.*, 1992), and in body fluids (Zhu *et al.*, 1991; Molzer *et al.*, 1992). The fatty acid pattern of sulfatides is dependent on the developmental stage and on the tissue. The fact that in MLD each tissue accumulates the corresponding normal fatty acid pattern of structurally normal sulfatides supports the idea of an independent accumulation in each tissue (Svennerholm *et al.*, 1962; Malone *et al.*, 1966).

Sulfatides are localized on the extracellular surface of the myelin sheath (Norton *et al.*, 1973; Arvanitis *et al.*, 1992) and are responsible for the preservation of the insulator function of myelin (Bosio *et al.*, 1996; Coetzee *et al.*, 1996).

To determine the importance of the main glycolipids in myelin (galactocerebroside and sulfatide), a mouse model was generated that lack both glycolipids (UDP-galactose:ceramide galactosyltransferase (CGT) deficient mice), (Coetzee *et al.*, 1996). These mice do not have a dramatic inhibition of myelination, as it could be expected, but they present an abnormal central nervous system myelin with

ultrastructural alterations that seem to reflect abnormal axo-glia adhesion/ recognition mechanisms (Marcus *et al.*, 2002a) and they develop progressive neuropathological symptoms leading to early death (Coetzee *et al.*, 1996). The myelin glycolipids and the myelin-associated glycoproteins seem to act together to promote myelin axo-glial adhesion and structural maintenance of the paranode (Marcus *et al.*, 2002a; Marcus *et al.*, 2002b). A mouse model lacking cerebroside sulfotransferase activity was also recently generated (Honke *et al.*, 2002). These mice, which lack sulfatide in brain and seminolipid in testis, develop neuropathological symptoms and, in spite of the preservation of compact myelin, they have abnormalities in paranodal junctions. Sulfoglycolipids thus seem to have an important role in myelin function and spermatogenesis (Honke *et al.*, 2002).

Sulfatides also seem to be important for several other biologic processes like, for example, the active sodium transport (as a cofactor for $Na^+$/ $K^+$-ATPase), the binding of opiates, GABA and serotonin to their receptors, the modulation of the activity of enzymes such as phospholipase A2, calmodulin-dependent cyclic nucleotide phosphodiesterase and phosphatidylinositol phosphate 3-kinase, the specific binding of several cellular adhesion molecules or the activation of several clotting substances (see von Figura *et al.*, 2001 for review). Sulfatides are expressed in the platelet surface and seem to have a role in platelet adhesion and aggregation by modulation of platelet activation (Merten *et al.*, 2001; Devaiah *et al.*, 2000). It is also known that sulfatides bind L-selectin, a lymphocyte-homing receptor (Suzuki *et al.*, 1993) that seems to modulate lymphocyte activation during cell adhesion and transmigration (Ding *et al.*, 2003), and several infectious organisms also have specific domains for sulfatide binding (Brennan *et al.*, 1991; Cerami *et al.*, 1992; Muller *et al.*, 1993; Olson *et al.*, 1993).

Sulfatides seem also to be involved in controlling insulin secretion, in stabilising insulin hexamer crystals and in mediating the conversion of insulin hexamers to the biological active monomers (Buschard *et al.*, 2002; Osterbye *et al.*, 2001). It has also been shown that insulin induces cerebroside sulfotransferase in oligodendrocyte's cell cultures (Ferret-Sena *et al.*, 1990). The physiological significance of sulfatides' function in all these mechanisms is still unknown.

The other three sulfated glycolipids represented in Figure 2 are less important in MLD.

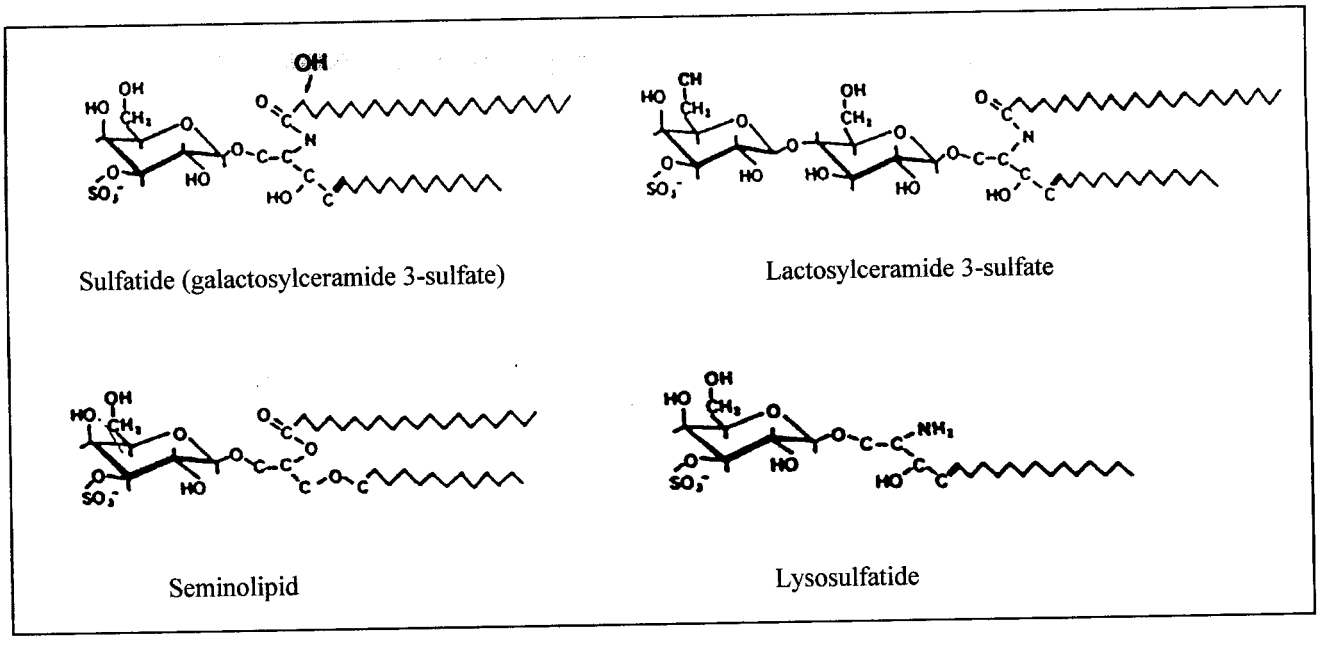



**Figure 2 – Sulfated glycolipids important for MLD.** In spite of the recent identification of other sulfated glycolipids in different tissues, only sulfatides, lactosylceramide 3-sulfate and seminolipid have been shown to be desulfated by ARSA in humans. Sulfatides are most important in central and peripheral nervous system, but they are also abundant in the kidney. Lactosylceramide 3-sulfate is also found in human kidney and urine, but it is not present in the brain. Seminolipid is the major sulfolipid in mammalian testes and sperm and it also could not be found in brain. Lysosulfatide is a highly cytotoxic compound that has been reported to accumulate in MLD, however the enzymatic deacylation of sulfatide to lysosulfatide was never proven (modified from von Figura *et al.*, 2001).

Lactosylceramide 3-sulphate can also be found in the kidney (Martensson *et al.*, 1966), lysosulfatide occurs in small amounts in the cerebral white matter, spinal cord and kidney (Toda *et al.*, 1990) and seminolipid is the major glycolipid constituent of mature mammalian testes and sperm (Kornblatt *et al.*, 1974). Sandhoff *et al.* (2002) have shown that a more complex sulfatide, gangliotetraosylceramide 3, 3-bis-sulfate also accumulates in the ARSA-deficient mice kidney, together with galactosylceramide 3-sulphate and lactosylceramide 3-sulphate. The authors thus suggested that in the murine kidney, ARSA is probably the only sphingolipid-sulfatase cleaving the galactosyl-3-sulfate bond. The analysis of the lipids accumulating in the kidney of mice models for several glycosphingolipid disorders also allowed the authors to suggest that the human pathway for complex sulfatides is very similar to that of mice.

### 1.5.2. Biosynthesis and degradation of sulfatide

Sulfatides result from the sulfation of galactosylceramide by reaction with 3'-phosphoadenosine-5'-phosphosulfate (PAPS), in a reaction catalysed by a microsomal sulfotransferase (Farrell *et al.*, 1971). They account for 3.5-4% of the total lipids of myelin (Norton *et al.*, 1982). Its synthesis is maximal at the myelination period and can occur in neurons, oligodendroglial cells, cells of glial origin and cells from the renal tubule epithelium (Benjamins *et al.*, 1974; Ishizuka *et al.*, 1978).

The degradation of 3-sulfogalactosyl-containing glycolipids begins with its desulfation by the lysosomal enzyme ARSA, in a reaction dependent on Saposin B (Mehl *et al.*, 1965; Shapiro *et al.*, 1979). The proposed mechanism for this reaction is referred in the next section of this chapter.

## 1.6. Proteins involved in sulfatide hydrolysis: Arylsulfatase A

### 1.6.1. Arylsulfatase A gene

The ARSA gene has been mapped to the chromosome 22q13 (Bruns *et al.*, 1978; Phelan *et al.*, 1992). A genomic clone containing the entire human ARSA gene was isolated and characterised in 1990 (Kreysing *et al.*, 1990). The ARSA gene consists of eight exons distributed along 3,2 Kb (GeneBank sequence X52150). Its promotor has no typical TATA or CAAT box sequences, but four major transcription initiation sites were identified between positions –367bp and –387bp, upstream of the start codon. As it is characteristic of housekeeping gene promoters, besides the lack of the TATA box, the ARSA promoter has a high GC content, with several GC boxes in typical positions for interaction with the transcription factor Sp1, and a CpG island (Kreysing *et al.*, 1990).

### 1.6.2. Arylsulfatase A mRNA

The ARSA cDNA hybridises to three different mRNA species (2.1 Kb, 3.7 Kb and 4.8 Kb) that differ in their 3' untranslated sequences, probably due to the use of different polyadenylation signals. The primary transcript is cleaved and polyadenylated 95 nucleotides after the termination codon, originating the 2.1 Kb mRNA. This transcript accounts for 90% of ARSA polyadelylated mRNA. The low ARSA activity resulting from a polymorphism destroying this polyadenylation signal suggests that the translational efficiency of the other transcripts is very low (Gieselmann *et al.*, 1989).

### 1.6.3. Arylsulfatase A protein

A full-length cDNA for human ARSA was cloned and sequenced in 1989 (Stein *et al.*, 1989). It has an open reading frame of 1521 nucleotides that codes for 507 amino

acids, from which the first 18 constitute a typical sequence for translocation into the ER. The authors reported that the ARSA does not seem to be subjected to any process of maturation by proteolytic processing in the lysosome.

The predicted ARSA sequence contains three potential N-glycosylation sites (Asn158, Asn184, Asn350). All three sites can be used and the oligosaccharides seem to be phosphorylated independently of their position. However the Asn184 seems to be less efficiently phosphorylated. The first and second sites, both encoded in exon 3, seem to be mutually exclusive and glycosylation in one of these sites and in site three (exon 6), seems to be sufficient for correct sorting of ARSA (Gieselmann *et al.*, 1992).

In human fibroblasts, ARSA is synthesised as a precursor with a mean apparent molecular mass of 62 KDa, which is slowly converted in the lysosome to a 60.5 KDa form. This conversion is thought to be due to oligosaccharides' trimming and the 60.5 KDa form is thought to represent the mature form. Just about 90% of the ARSA forms seem to contain two side chains of the high-mannose or hybrid type oligosaccharides; the other 10% contain probably one complex and one high-mannose oligosaccharide (Waheed *et al.*, 1982). Heterogeneity for carbohydrate composition in different tissues can be observed as was also reported for other lysosomal enzymes (Hasilik *et al.*, 1982).

### 1.6.4. Arylsulfatase A three dimensional structure

The three dimensional structure of human ARSA, based on X-ray diffraction data to 2.1 Å resolution, was reported by Lukatela *et al.* (1998). In acidic milieu, characteristic for lysosomes, ARSA exists as an octamer that at neutral pH dissociates into dimers. The ARSA monomer has a globular hat-like structure. Two monomers associate through contacts involving one β-strand and four loops localised on the "flat"

part of the hat-like structure. These contacts are made through direct hydrogen bonds and water-mediated hydrogen bonds, which originate a very strong monomer-monomer interaction. In the homodimer, which presents an almost cylindrical form, the two monomers are related by a crystallographic 2-fold axis (Lukatela *et al.*, 1998).

The octamers, observed at acidic pH, are composed of dimers $(\alpha_2)_4$ and seem to be stabilised by hydrophobic interactions. The pH dependent dimer-octamer association is due to protonation of Glu424. The amino acid residues Cis-Pro425, Pro426, Phe398, Phe399 and Ala422, form an hydrophobic cavity that encloses Glu424 and several water molecules. At acidic pH, the Glu424 residue is protonated and establishes an intermolecular hydrogen bond with the oxygen of Phe398, thus stabilizing the octamer. At neutral pH, this hydrogen bond is no longer possible, and thus the octamer is destabilized by electrostatic repulsion between the negatively charged Glu424 side chains (Lukatela *et al.*, 1998).

The secondary structure of the enzyme is of the α/β type (see Figure 3) and the enzyme consists of two domains. One domain includes the N-terminal region and contains a large central beta-sheet, flanked by alpha-helices. The other domain, which includes the C-terminal region, is smaller and consists of a small antiparallel beta-sheet tightly associated with a long C-terminal alpha-helix. The two beta-pleated sheets are connected by indirect hydrogen bonds through several buried water molecules and covalently linked by disulfide Cys300-Cys414 (Lukatela *et al.*, 1998).

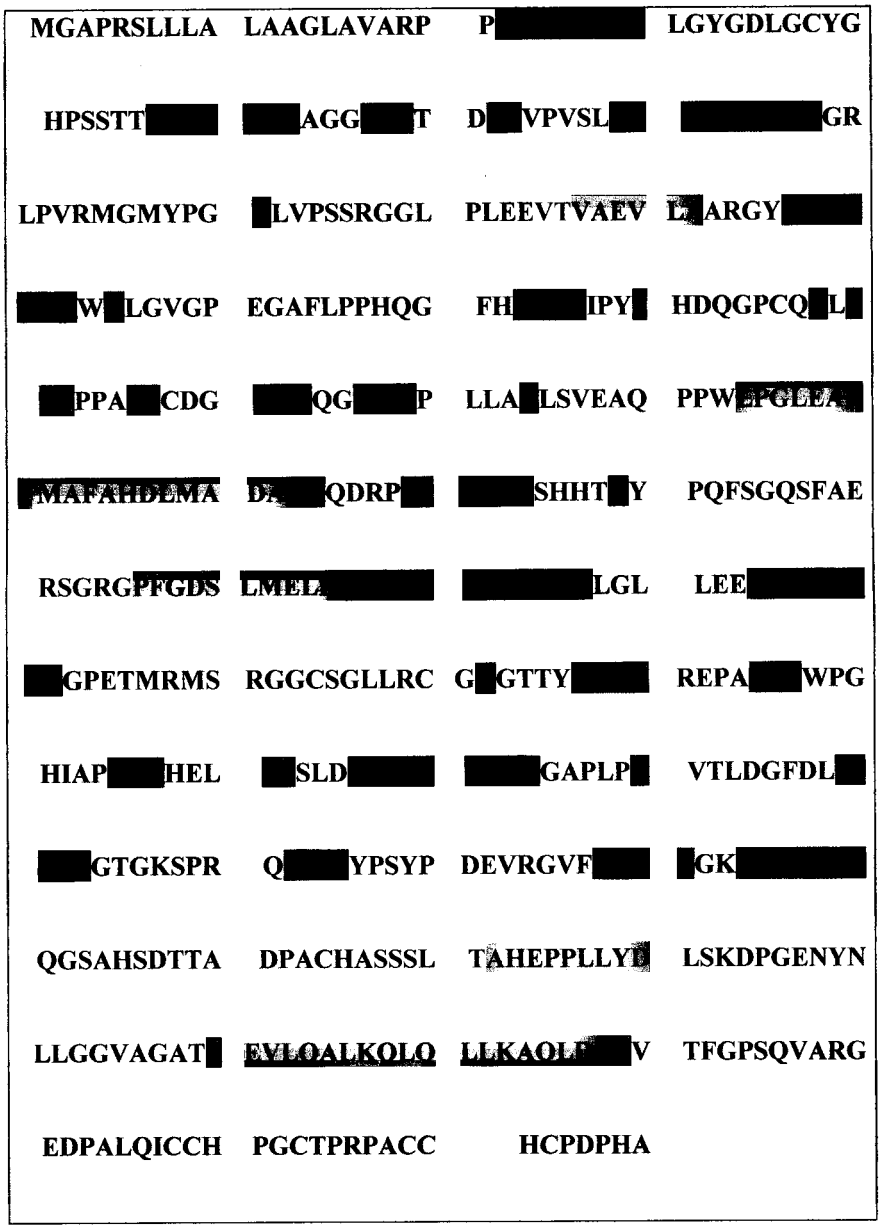



**Figure 3 - Secondary structure of ARSA.** Alpha-helices are indicated in blue and beta-strands are indicated in grey. Putative amino acid residues in the active site and glycosylation sites are indicated in green and red, respectively (adapted from Waheed *et al.*, 1982; Lukatela *et al*, 1998 and Waldow *et al.*, 1999).

### 1.6.5. Arylsulfatase A active site

The catalytic site of ARSA includes the conserved FGly residue, critical for sulfatases activity, at position 69, where the cDNA sequence predicts a cysteine (Lukatela *et al.*, 1998). The sequence information essential for the cysteine conversion in sulfatases is contained in a stretch of amino acid residues from positions –1 to +11 regarding the cysteine to be modified. In ARSA, two sequence motifs were identified within this region: a CTPSR motif, starting with Cysteine 69, and a second auxiliary AALLTGR motif, including residues from position 74 to 80. Apart of the modified cysteine, and in spite of its high conservation, no other residue seems to be strictly essential. However, systematic substitution of the amino acid residues in the CTPSR motif demonstrated that the cysteine, proline and arginine are key residues. The serine and threonine can be individually, but not simultaneously substituted without interfering in the cysteine oxidation (Dierks *et al.*, 1999). The importance of the auxiliary motif seems to be related with its secondary structure (Schmidt *et al.*, 1995; Dierks *et al.*, 1999).

The FGly residue, that is essential for the enzyme catalytic activity, is localised in the bottom of a positively charged cavity that constitutes the active binding site for the negatively charged sulfate group of the substrate. This residue is involved in the coordination of an $Mg^{2+}$ metal ion, together with amino acid residues Asp29, Asp30, Asp281 and Asn282. All these residues are conserved among sulfatases, except for Asn282 that can be replaced by Gln or His, residues that also have metal coordination capacity. All these residues are important for the general structure of the active site (Waldow *et al.*, 1999). Lys302, His229, Ser150, Arg73, His125 and Lys123 are the other putative active site residues, and they are thought to be involved in the ARSA

catalytic mechanism. All the residues involved in sulfate binding or in catalysis have the capacity to establish hydrogen bonds, originating a net that constitute the ARSA functional catalytic system (Waldow *et al.*, 1999).

### 1.6.6. Arylsulfatase A reaction mechanism

Based on the ARSA crystal structure and on its high degree of structural similarity with the *E. coli* alkaline phosphatase, Lukatela *et al.* (1998) proposed a catalytic mechanism for ARSA that includes a nucleophilic attack on the sulfate sulfur atom, by one of the hydroxyls of the FGly, and a formation of a covalent intermediate. The functions of nine conserved putative active site amino acid residues in ARSA (Asp29, Asp30, Asp281, Asn282, His125, His229, Lys123, Lys302 and Ser150) were analysed by Waldow *et al.* (1999), in a study in which each of these residues was conservatively substituted and the Km, Vmax and pH optimum of the mutants toward the artificial substrate p- nitrocatechol sulfate determined. According to the authors each of these residues has a critical function for ARSA catalytic activity. However, the fact that ARSA degrades its substrate very fast makes it difficult to characterise the intermediate complexes. More recently, von Bülow *et al.* (2001) reported the crystal structure of two ARSA mutants, Cys69Ala and Cys69Ser-ARSA, in complex with p-nitrocatechol sulfate. For the first mutant there is no possibility of formation of the covalent substrate-enzyme intermediate, in spite of the preservation of the geometry and polarity of the active site environment. For the second mutant the catalysis can occur, but the release of the sulfate from the enzyme is blocked, which allows the characterisation of the sulfate intermediate. According to the authors, the sulfate group binds the enzyme through hydrogen bonds with the positively charged side chains of

His229 and Lys302. Weaker bonds are also established with the side chains of Lys123 and Ser150, and to the main chain nitrogen of residue Cys69 and $Mg^{2+}$. The sugar residue of the sulfatide, the ARSA natural substrate, could eventually substitute the hydroxyl groups of p-nitrocatechol sulfate in the interaction with the charged side chains of Arg288 and Asp152. The side chains of His405, Glu155 and Asp173 are also probably involved in interactions between the enzyme and the sulfatide sugar residue. The amino acid residues Leu68, Val91 and Val93 can yet stabilize the positioning of the sulfatide hydrophobic chains through hydrophobic interactions (von Bülow *et al.*, 2001).

The proposed mechanism for sulfatide degradation by ARSA is depicted in Figure 4. In the resting form of ARSA, the FGly group is probably hydrated and acting as a geminal diol. One oxygen, from one of the hydroxyls, can start a nucleophilic attack on the sulfur atom initiating the hydrolysis via a transesterification step, and thus originating a covalent intermediate. The second hydroxyl can eliminate the sulfate through a C-O cleavage and regenerate the aldehyde, whose hydration completes the catalytic cycle (Lukatela *et al.*, 1998; von Bülow *et al.*, 2001).

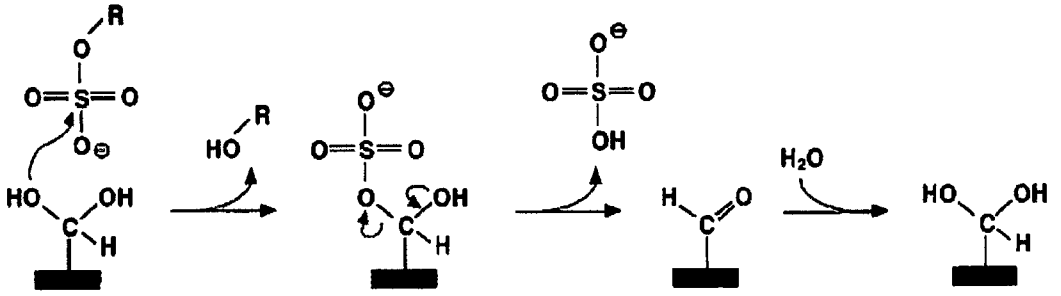


**Figure 4 – Proposed mechanism for the hydrolysis of sulfatide by ARSA.** The FGly group acts like a geminal diol. The sulfur atom suffers a nucleophilic attack from one of the hydroxyls and an enzyme-sulfate ester intermediate forms, while a residual alcohol is released. The sulfate is then eliminated by an intramolecular rearrangement that cleaves the ester bond and regenerates the aldehyde. The hydration of the aldehyde completes the catalytic cycle (modified from Waldow *et al.*, 1999).

## 1.7. Proteins involved in sulfatide hydrolysis: Saposin B

The small protein Saposin B results from the proteolytic processing of prosaposin, a 524 amino acid protein, which is composed of four homologous domains. Each of these domains generates, by lysosomal proteolytic processing, a mature independent glycoprotein: SAP-A, SAP-B, SAP-C and SAP-D. The SAP precursor gene covers a region of 17Kb in chromosome 10q21-23 (for review see Sandhoff *et al.*, 2001).

Saposin B acts by extracting specific lipids from membranes and forming soluble activator-lipid complexes that are recognized by ARSA. There is no interaction between the activator and the enzyme (Sandhoff *et al.*, 2001). The crystal structure of human Saposin B was recently reported and revealed an unusual shell like structure dimer that can be important to do the extraction of target lipids from membranes (Ahn *et al.*, 2003a). Stability studies revealed that it is extremely resistant to pH, heat and

proteases, which can be related with three intrachain disulfide bridges that are part of its structure (Ahn *et al.*, 2003b).

## 1.8. Arylsulfatase A pseudodeficiency

### 1.8.1. Definition of Arylsulfatase A-pseudodeficiency

Arylsulfatase A-pseudodeficiency (ARSA-PD) is a relatively frequent condition among the general population (1-2%), which is characterised by a reduction to 5-15% of ARSA activity against artificial substrates. However, the "in vivo" activity of this altered ARSA is sufficient to prevent the accumulation of sulfatide and the development of a MLD phenotype (reviewed in von Figura *et al.*, 2001).

### 1.8.2. Molecular lesions in the origin of ARSA-PD

Gieselmann *et al.* (1989) identified the molecular defects in the origin of ARSA-PD, they consist of two A→G transitions in the ARSA coding gene (nucleotides 2417 and 3352, according to the GeneBank sequence X52150), which can be found isolated or in combination with each other. The first mutation changes asparagine 350 into serine and causes the abolition of one ARSA glycosylation site, which explains the smaller size of ARSA in pseudodeficiency (2-4 KDa less). The second mutation destroys the first polyadenylation site downstream of the stop codon, leading to the loss of the 2.1 Kb mRNA species that normally represents 90% of the ARSA polyadenylated mRNA. The composed allele, with both alterations, is usually known as the ARSA-PD allele. In this work, and in spite of the existing controversy regarding the amount of ARSA activity associated with the two alleles with only one of the ARSA-PD

associated alterations, we also refer to these alleles as low ARSA activity alleles or ARSA-PD associated alleles.

### 1.8.3. Biochemical characterisation of the ARSA-PD protein

According to Gieselmann *et al.* (1989) the loss of the major ARSA mRNA species, due to the destruction of the polyadenylation site, can by it self explain the 90% reduction in the synthesis of ARSA, which results in the low ARSA activity associated with the complex ARSA-PD allele in human fibroblasts. This observation is not confirmed by Harvey *et al.* (1998) that reported a reduction of only 69% in the ARSA mRNA due to the polyadenylation defect.

There were also some contradictory observations regarding the kinetic properties of the ARSA-PD protein. Qu *et al.* (1997/ 1998), performed kinetic studies to compare the hydrolysis of p-nitrocatechol sulfate by ARSA, partially purified from normal fibroblasts or from fibroblasts of a homozygote for the ARSA-PD allele. They reported for the ARSA-PD protein a decreased affinity for the substrate and an increased heat inactivation. On the contrary, Chang and Davidson (1983) and Herz and Bach (1984) had compared the properties of ARSA from normal fibroblasts and from MLD/ PD fibroblasts, and they observed identical kinetic properties, substrate affinity, optimal pH for activity, and sensitivity to heat denaturation and proteolytic degradation, for the hydrolysis of 4-methylumbelliferyl sulfate and p-nitrocatechol sulfate, respectively.

The biochemical characteristics of the N350S-ARSA protein have also been the subject of contradictory reports. Its rate of synthesis was found to be normal in BHK transfected cells expressing the N350S-ARSA protein (Gieselmann *et al.*, 1989) and in human fibroblasts of N350S homozygotes (Park *et al.*, 1996). Its specific activity for

artificial substrates was also found to be normal by Gieselmann *et al.* (1989), Ameen *et al.* (1990) and Park *et al.* (1996). Only Harvey *et al.* (1998) reported a contribution of this alteration to the reduced activity of the ARSA protein, when expressed in CHO transfected cells. According to these authors there is also a deficient intracellular localization of N350S-ARSA, with 55% of the enzyme localizing to non-lysosomal fractions in a density gradient. On the contrary, Ameen *et al.* (1990) and Park *et al.* (1996) reported a normal intra-lysosomal localization for N350S-ARSA. Gieselmann *et al.* (1989) also reported a normal stability for this protein expressed in BHK transfected cells. However, Ameen *et al.* (1990) and Park *et al.* (1996) reported a decreased stability of this enzyme in human fibroblasts.

The measure of ARSA activity using artificial substrates, in N350S homozygotes or heterozygotes, also resulted in contradictory observations. Barth *et al.* (1994) and Leistner *et al.* (1995) reported that the N350S alteration is not responsible for a major reduction in ARSA activity in human leukocytes. On the contrary, Shen *et al.* (1993) and Park *et al.* (1996) observed a reduction in the N350S-ARSA activity in human fibroblasts.

### 1.8.4. Clinical implications of ARSA-PD

The relatively high frequency of ARSA-PD in the general population could be explained by an heterozygote advantage, but to date there are no plausible possibilities for this advantage. On the other hand, many studies have been done trying to elucidate the existence of clinical implications for ARSA-PD, namely its contribution to an increased susceptibility to the development of psychiatric or neurological symptoms. Nevertheless, most of these studies were based on the biochemical determination of

ARSA activity "in vitro" or included a small number of subjects and lead to inconclusive results (for review see von Figura *et al.*, 2001).

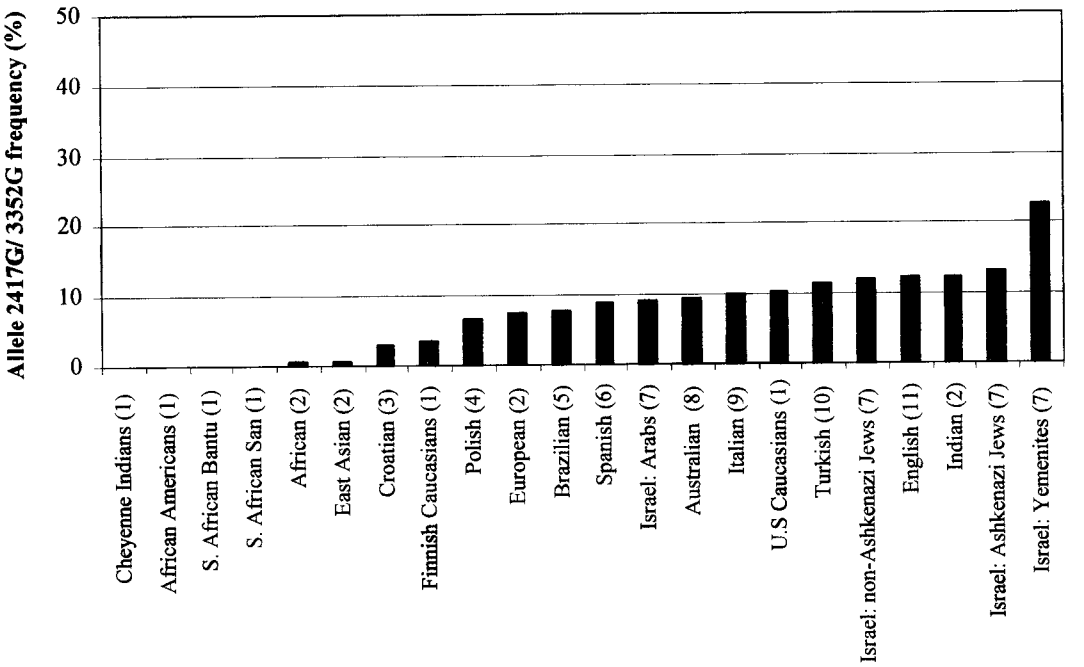
Conzelmann and Sandhoff (1984) proposed a theoretical model to explain the relationship between a residual enzyme activity and the development of lysosomal storage diseases. According to this model there is a critical threshold value for the residual activity necessary to maintain a steady state substrate concentration within the lysosome. When the residual enzyme activity falls below this level, the substrate starts to accumulate at a constant rate, which depends linearly on the amount of residual activity. This model could explain that for individuals with a low ARSA activity, close to the threshold limit, a small variation in ARSA activity in the lysosome, even if temporary, could lead to an irreversible accumulation of sulfatide with possible clinical implications.

### 1.8.5. Origin and frequency of low ARSA activity alleles

The low ARSA activity alleles 2417G/ 3352G, 2417G/ 3352A and 2417A/ 3352G have very different frequencies in different populations (see graphics 1 and 2). The 2417G/ 3352A allele seems to be very frequent, presenting frequencies as high as 44% in some South African tribes (Ricketts *et al.*, 1996). On the contrary, the allele 2417A/ 3352G seems to be very rare, since only five non-related cases have been described worldwide (Barth *et al.*, 1994; Leistner *et al.*, 1995; Ricketts *et al.*, 1996; Gort *et al.*, 1999). Interestingly, the frequencies of alleles 2417G/ 3352G and 2417G/ 3352A seem to be inversely correlated in Africans/ Asians (high frequency of 2417G/ 3352A allele, 2417G/ 3352G allele almost non-existing) and Europeans/ Indians, with a similar

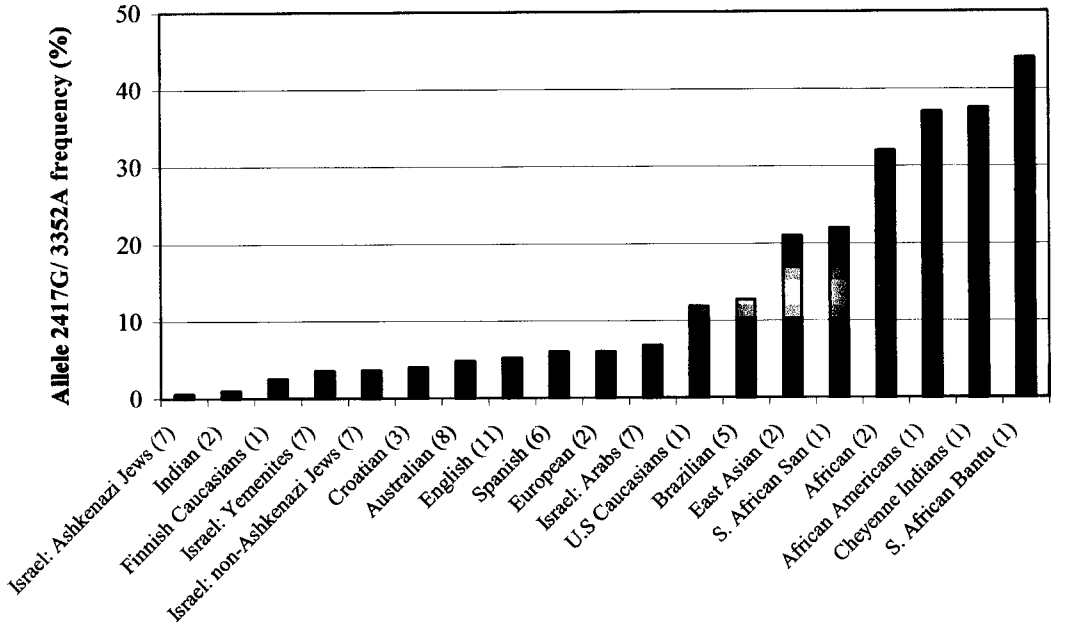
high combined frequency in both groups of populations (Ott *et al.*, 1997). Once again, this could indicate some selective advantage for low ARSA activity alleles.

Frequency determination and haplotype analysis in different populations raised several hypotheses regarding the order of appearance of the alterations that characterise low ARSA activity alleles and its appearance as single or recurrent events (Zlotogora *et al.*, 1994a; Ott *et al.*, 1997).




**Graphic 1 – Frequency of ARSA allele 2417G/ 3352G.**

(1) Ricketts *et al.*, 1996 (2) Ott *et al.*, 1997 (3) Bognar *et al.*, 2002 (4) Lugowska *et al.*, 2000 (5) Pedron *et al.*, 1999 (6) Gort *et al.*, 1999 (7) Zlotogora *et al.*, 1994a (8) Nelson *et al.*, 1991(9) Regis *et al.*, 1996 (10) Emre *et al.*, 2000 (11) Barth *et al.*, 1994.




**Graphic 2 – Frequency of ARSA allele 2417G/ 3352A.**

(1) Ricketts *et al.*, 1996 (2) Ott *et al.*, 1997 (3) Bognar *et al.*, 2002 (5) Pedron *et al.*, 1999 (6) Gort *et al.*, 1999 (7) Zlotogora *et al.*, 1994a (8) Nelson *et al.*, 1991 (11) Barth *et al.*, 1994.

The doubly mutated ARSA-PD allele seems to be associated with a highly conserved haplotype, which together with its high frequency in Europeans and its absence in non-Europeans, strongly suggests a single ancient origin posterior to the divergence of European and Asian populations. The occurrence of the N350S mutation in this haplotype would have had to occur first and before the divergence of the African and non-African lineages. In the case of the singly mutated alleles, their identification on divergent haplotypes suggests the existence of "de novo" mutation events (Ott *et al.*, 1997).

## 1.9. Molecular characterisation of MLD

At the molecular level, MLD is very heterogeneous. Approximately ninety MLD-causing mutations have been described in the ARSA gene (von Figura *et al.*, 2001 and *www.hgmd.org.*), most of these mutations being present in a single family. Missense mutations are the most frequent type of alterations in the ARSA gene. Three nonsense mutations, ten small deletions, one inversion and seven nucleotide substitutions originating altered splicing were also described. To date, no gross alterations were described in this gene and none of the mutations reported so far affects the amino acid residues thought to be part of the enzyme's active site (see Figures 3, 5 and 6 and Table 1). Several amino acid substitutions in the ARSA gene were found to be polymorphic, they do not lead to a decrease in the enzyme activity and they can be found in the general population; examples of these are alterations L76P (Berger *et al.*, 1996), W193C (Polten *et al.*, 1991), T391S (Polten *et al.*, 1991), A464V (Berger *et al.*, 1999), and R496H (Ricketts *et al.*, 1998). This fact clearly elucidates the need to prove the causality of new mutations. In most cases, expression studies were performed to exclude the possibility of novel mutations being polymorphisms, but only in few cases the mutated enzymes were further characterised (see Figure 6).

Several mutations have been described in the background of the ARSA-PD alleles (Zlotogora *et al.*, 1995; Regis *et al.*, 1998; Gieselmann *et al.*, 1991). Thus, the genetic determination of ARSA-PD alleles can be helpful as a complement of the biochemical diagnosis of MLD, but care has to be taken because the presence of ARSA-PD mutations does not exclude the presence of causal MLD mutations.

Three different mutations seem to be relatively frequent among Europeans: mutation IVS2+1G>A, which is the causal mutation of allele I, mutations P426L (allele A) and I179S.

The allele I (Polten *et al.*, 1991) is characterised by four different alterations. Two of these substitutions (W193C and T391S) were shown to be polymorphisms that can be found in healthy individuals and whose introduction in ARSA cDNA does not affect the enzyme activity. Another alteration in intron 7 (g2842c, GeneBank sequence X52150) can also be found in normal individuals. The fourth alteration (IVS2+1G>A) destroys the splice donor site at the 3' exon/ intron border of exon 2. Patients homozygous for this alteration do not produce ARSA mRNA. The three polymorphic alterations define an haplotype that is in complete linkage disequilibrium with the causal alteration, thus raising the possibility of a common origin for this allele (Zlotogora *et al.*, 1994b). Allele I presents different frequencies in different European populations of MLD patients (25 - 43%), (Polten *et al.*, 1991; Gieselmann *et al.*, 1991; Barth *et al.*, 1993a; Regis *et al.*, 1996; Gort *et al.*, 1999).

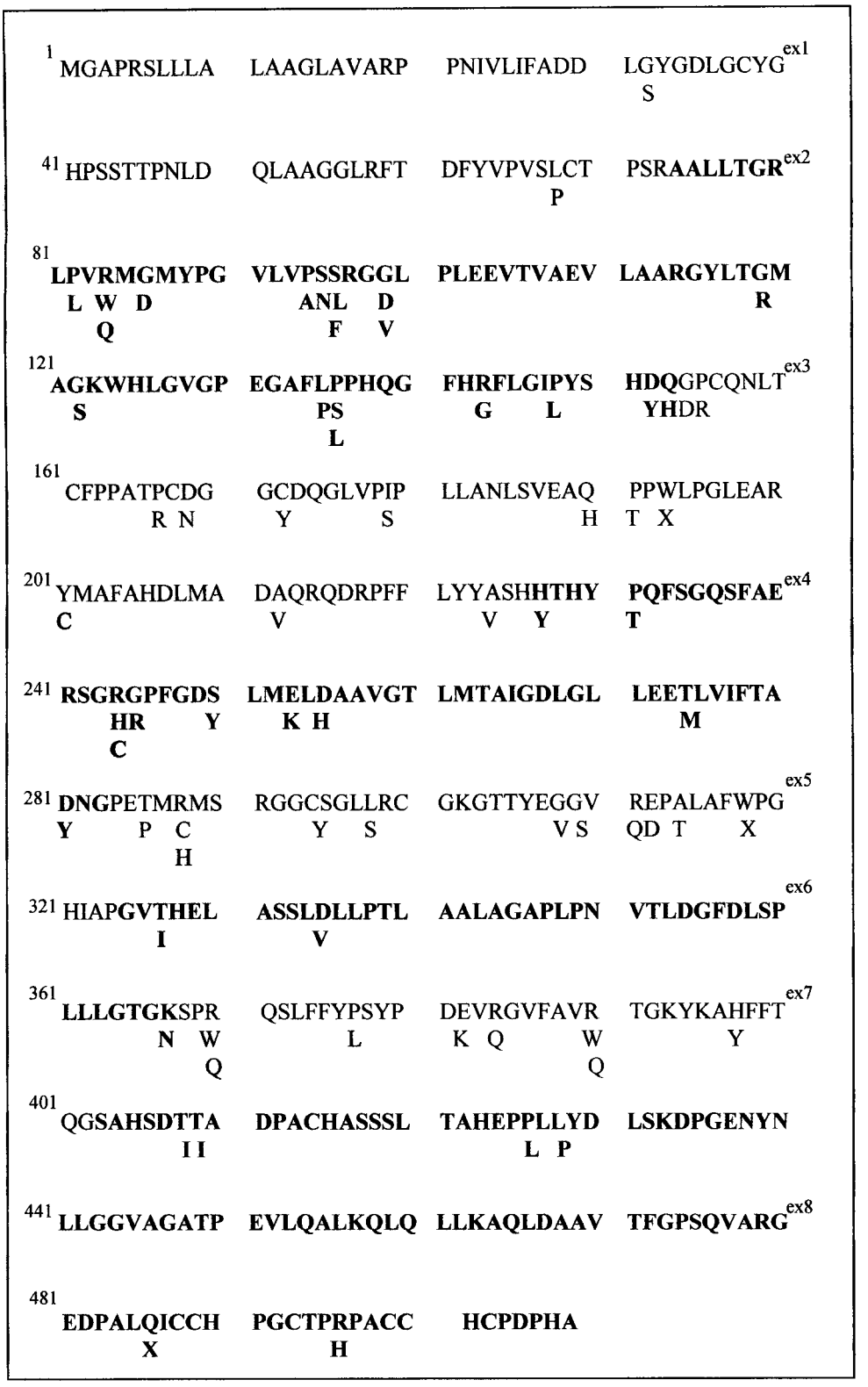



**Figure 5 - Amino acid substitutions reported in the ARSA polypeptide.** The exons with an even number are represented in bold. Amino acid numbering is indicated in the left (adapted from von Figura *et al.* (2001) and *www.hgmd.org).*

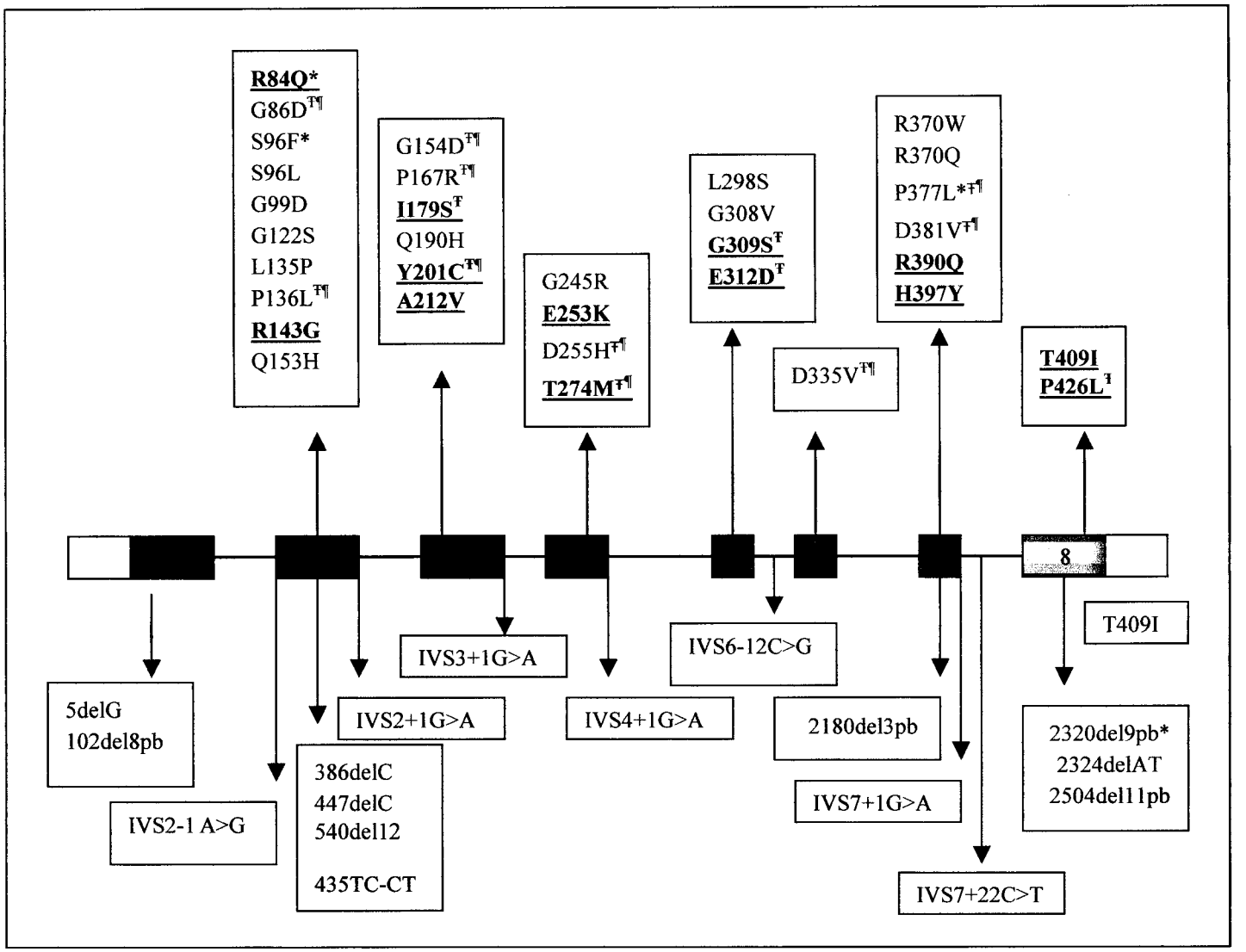



**Figure 6 – Mutations in the ARSA gene.** Distribution along the ARSA gene of the amino acid substitutions resulting from missense mutations proved to be causal by expression studies (upper part of the ARSA gene picture) and of the small deletions and alterations leading to splicing alterations (lower part of the ARSA gene picture). Missense mutations originating an enzyme with residual activity are underlined. *Mutations that were found in the ARSA-PD allele background. [T]Mutations that were further characterised, all these mutations were found to originate enzymes with a reduced half-life. [¶]Mutations originating the protein's retention in the ER. More detailed description and references for these mutations can be found in Table 1.

## Table 1 - Mutations reported in the ARSA gene.

| Mutation | Exon | Sequence alteration | Population | Reference |
|---|---|---|---|---|
| **Missense mutations proved by expression studies** | | | | |
| R84Q[a] | Exon 2 | 400G>A | European | Kappler, 1992; Berger, 1997 |
| G86D | Exon 2 | 406G>A | Muslin Arab | Heinish, 1995; Hermann, 2000 |
| S96F[a] | Exon 2 | 436C>T | European | Gieselmann, 1991; Berger, 1997 |
| S96L | Exon 2 | 435/436TC>CT | Muslin Arab | Heinish, 1995 |
| G99D | Exon 2 | 445G>A | Japanese | Kondo, 1991; Eto, 1993 |
| G122S | Exon 2 | 513G>A | Japanese/ European | Honke, 1993; Kappler, 1994 |
| L135P | Exon 2 | 553T>C | European | Gomez-Lira, 1998 |
| P136L | Exon 2 | 559C>T | Jewish | Kafert, 1995 |
| R143G | Exon 2 | 576C>G | Vietnam | Arbour, 2000 |
| Q153H | Exon 2 | 608G>C | Japanese | Tsuda, 1996 |
| G154D | Exon 3 | 724G>A | European | Kappler, 1994 |
| P167R | Exon 3 | 763C>G | European | Kappler, 1994 |
| I179S | Exon 3 | 799T>G | European | Fluharthy, 1991 |
| Q190H | Exon 3 | 833G>C | Muslin Arab | Heinish, 1995 |
| Y201C | Exon 3 | 865A>G | European | Hermann, 2000 |
| A212V | Exon 3 | 898G>T | European | Barth, 1993b; Coulter-Mackie, 1997a |
| A224V | Exon 3 | 934C>T | European | Barth, 1993b |
| G245R | Exon4 | 1070G>A | Japanese | Hasegawa, 1993 |
| E253K | Exon4 | 1094G>A | European | Regis, 2002 |
| D255H | Exon4 | 1100G>C | European | Lissens, 1996 ; Hermann, 2000 |
| T274M | Exon4 | 1158C>T | Lebanese | Harvey, 1993; Hess, 1996a |
| S295Y | Exon 5 | 1533C>A | European | Barth, 1993b |
| L298S | Exon 5 | 1542T>C | Japanese | Kurosawa, 1998 |
| G308V | Exon 5 | 1572G>T | Japanese | Tsuda, 1996 |
| G309S | Exon 5 | 1574G>A | European | Kreysing, 1993 |
| E312D | Exon 5 | 1585G>T | European | Hermann, 2000 |
| D335V[b] | Exon 6 | 1743A>T | European | Hess, 1996a; Schestag, 2002 |
| R370W[b] | Exon 7 | 2093C>T | Christian Arab | Heinish, 1995; Schestag, 2002 |
| R370Q[b] | Exon 7 | 2094G>A | Jewish | von Figura, 2001; Schestag, 2002 |
| P377L[a] | Exon 7 | 2119C>T | Habbanite Jewish | Zlotogora, 1995 |
| D381V | Exon 7 | 2131G>A | European | von Figura, 2001 |
| E382K | Exon 7 | G2133>A | European | Barth, 1993b |
| R390Q | Exon 7 | 2158G>A | unknown | Coulter-Mackie, 1998 |
| H397Y | Exon 7 | 2178C>T | unknown | Coulter-Mackie, 1998 |
| T409I | Exon 8 | 2330C>T | Japanese | Hasegawa, 1994 |
| P426L | Exon 8 | 2381C>T | European | Polten, 1991 |

| Mutation | Exon/ Intron | Sequence alteration | Population | Reference |
|---|---|---|---|---|
| **Nonsense mutations** | | | | |
| W193X | Exon 3 | 842G>A | European | Gort, 1999 |
| W318X | Exon 5 | 1603G>A | Vietnam | Arbour, 2000 |
| Q486X | Exon 8 | 2560C>T | European | Draghia, 1997 |
| **Small deletions and insertions** | | | | |
| 5delG | Exon 1 | ATGGgGGCA | European | Berger, 1999 |
| 102del8pb | Exon 1 | GGGgacctgggCTG | European | Draghia, 1997 |
| 386delC | Exon 2 | GGCcTGCCC | European | Gort, 1999 |
| 447delC | Exon 2 | GGCcTG | European | Kreysing, 1993 |
| 540del12pb | Exon 2 | TGaggggccttccTG | European | Luyten, 1995 |
| 2180del3pb | Exon 7 | CACttcTTC | European | Gieselmann, 1994 |
| 2320del9pb[a] | Exon 8 | CACagtgataccACT | European | Regis, 1998 |
| 2324delAT | Exon 8 | AGTGatACC | European | Regis, 1995 |
| 2504del11pb | Exon 8 | TTAGAcgcagctgtgaCC | European | Bohne, 1991 |
| 435TC-CT | Exon 2 | TC-CT | Arab | Heinish, 1995 |
| **Mutations originating splicing alterations** | | | | |
| IVS1-2$^{A}$>G | intron 1 | 366A>G | Japanese | Kurosawa, 1998 |
| IVS2+1G>A | intron 2 | 609G>A | European | Polten, 1991 |
| IVS3+1G>A | intron 3 | 942G>A | European | Barth, 1995 |
| IVS4+1G>A | intron 4 | 1186G>A | Navarro Indian/ | Pastor-Soler, 1994 |
| | | | Alaskan Eskimo | Pastor-Soler, 1995 |
| IVS6-12C>G | intron 6 | 2079G>C | European | Gort, 1999 |
| IVS7+1G>A | intron 7 | 2194G>A | European | Fluharthy, 1991 |
| IVS7+22C>T | intron 7 | 2215C>T | Japanese | Hasegawa, 1994 |
| T409I | exon 8 | 2330C>T | Japanese | Hasegawa, 1994 |

[a] Mutations identified in a ARSA-PD associated allele. [b] Residues Asp335 and Arg370 form an intramolecular salt bridge that is destroid by these mutations. Data according to von Figura *et al.* (2001) and *www.hgmd.org.*

The allele A (Polten *et al.*, 1991) is characterised by one single mutation in exon 8, which changes proline 426 into leucine (P426L). ARSA cross-reacting material and activity are absent in cultured fibroblasts of homozygous patients, but can be restored by treatment with a cysteine proteinase inhibitor (von Figura *et al.*, 1983; Polten *et al.*, 1991). This protein is synthesised in normal amounts, has enzymatic activity and is correctly targeted to the lysosome, where it is rapidly degraded (von Figura *et al.*, 1983; Gieselmann *et al.*, 1994). A recent publication by von Bülow *et al.* (2002), explains its instability in lysosomes by its defective oligomerization. This allele presents frequencies between 1.9 and 30% in different European populations of MLD patients and is frequently associated with late onset forms of MLD (Gieselmann *et al.*, 1991; Polten *et al.*, 1991; Barth *et al.*, 1993a; Regis *et al.*, 1996).

Mutation I179S represents 2-12% of the MLD causal mutations in Europe (Fluharty *et al.*, 1991; Berger *et al.*, 1997; Gomez-Lira *et al.*, 1998; Halsall *et al.*, 1999). The expression of an ARSA cDNA with this mutation, in BHK cells, produces only 5% of the enzyme activity measured from the expression of wild type ARSA (Fluharty *et al.*, 1991). This mutation was always observed in patients with late onset disease and interestingly homozygosity for this mutation was never reported (von Figura *et al.*, 2001).

Baumann *et al.* (2002), recently reported a study of 12 adult patients among whom they could distinguish two different adult clinical forms. Some patients presented mainly motor symptoms with central nervous system motor signs and peripheral neuropathy. Mutation P426L was frequently found in these patients. Other patients presented a psycho-cognitive form of the disease, with onset characterised by behavioural abnormalities including mood alterations, peculiar social reactions and

progressive mental deterioration. The neurological involvement could be observed only a few years after the onset. Among these patients, I179S was the most frequent mutation.

Some other mutations are relatively frequent among non-European populations. Kondo *et al.* (1991) reported mutation G99D in an adult Japanese patient. This mutation seems to represent 45% of the MLD causal alleles in Japan (Kurosawa *et al.*, 1998). Northern blotting analysis revealed a normal amount of ARSA mRNA in patients' fibroblasts. The introduction of this mutation in ARSA cDNA leads to a complete abolishment of ARSA activity (Kondo *et al.*, 1991).

Mutation IVS4+1G>A was found to be frequent among the Navajo Indian population and in the Southern Alaskan Eskimos (Pastor-Soler *et al.*, 1994; Pastor-Soler *et al.*, 1995). By Northern blotting analysis negligible amounts of ARSA mRNA were found, a deletion of all exon 4 with a new reading frame and a stop codon at exon 5, occurs.

Another mutation found with high frequency in a particular population is the mutation P377L, which was found in a PD allele background, in the Habbanite and Yemenite Jews communities (Zlotogora *et al.*, 1995). The ARSA protein with this mutation is retained in an early biosynthetic compartment and does not reach the lysosome (Gieselmann *et al.*, 1994).

In Australian and Australian Lebanese patients, mutation T274M represents 20% and 85% of the MLD causal mutations, respectively (Heinisch *et al.*, 1995; Harvey et *al.*, 1993). Low amounts of enzyme activity can be detected in cells expressing the T274M substituted cDNA. The mutant enzyme has a specific activity of 35% but the

majority of it seems to be rapidly degraded in an early biosynthetic compartment (Hess *et al.*, 1996a).

## 1.10. Genotype-Phenotype correlation in MLD

Polten *et al.* (1991) tried to establish a genotype-phenotype correlation in MLD. According to the authors, there are two kinds of alleles in MLD: null alleles and alleles that originate low amounts of residual enzyme activity (R alleles). Homozygosity for null alleles always cause the most severe late infantile form of the disease and two R-type alleles usually originate the milder adult form. One copy of an R allele should mitigate the course of the disease (Polten *et al.*, 1991).

In spite of this theory still being accepted in a general way, the initial hope that it would be possible to make straightforward genotype-phenotype correlations, predicting the clinical outcome from the genotype analysis, did not come through. Unknown factors, genetic or environmental, other than the ARSA gene mutations must contribute to the MLD phenotype. Not only is difficult to predict the clinical course of the disease in patients with one R allele, as they can present any of the three MLD clinical forms, but also intrafamilial heterogeneity was found regarding the age of onset and severity of the disease (for review see von Figura *et al.*, 2001).

## 1.11. Animal models for MLD

Animal models can be very important to the study of pathogenesis and approaches to therapy of lysosomal storage disorders.

The only animal model for MLD are the ARSA-deficient mice, generated by homologous recombination in embryonic stem cells (Hess *et al.*, 1996b). There are no

natural animal models. Complete ARSA activity absence and sulfatide storage in several organs, including the brain, could be found in the ARSA-deficient animals. Thus, biochemically they resemble the late infantile form of MLD. Various neurological and behavioural abnormalities can be observed in ARSA-deficient mice, but they have a phenotype much milder than it would be expected, with neurophatological alterations progressing slowly. This is probably related to the absence of demyelination in the central nervous system, although a variety of pathological alterations can be found in the nervous system. The mice phenotype thus seems to resemble early stages in the human disease; neuromotor alterations, impaired learning and memory and decline in brainstem auditory-evoked potentials could be observed (Hess *et al.*, 1996b; Gieselmann *et al.*, 1998; D'Hooge *et al.*, 1999a; D'Hooge *et al.*, 1999b; D'Hooge *et al.*, 2001; Schott *et al.*, 2001).

## 1.12. Therapy

Different potential therapeutic strategies have emerged recently for Lysosomal Storage Disorders (LSD). They are based on two major therapeutic approaches: enzyme supplementation, which can be achieved by enzyme replacement, bone marrow transplantation or gene therapy, and substrate deprivation.

Although enzyme replacement therapy is already approved or under clinical studies for several lysosomal storage disorders (Beutler *et al.*, 1997; Schiffmann *et al.*, 2000; Kakkis *et al.*, 2002; van den Hout *et al.*, 2001), this is an extremely expensive therapy and the administrated enzyme has no capacity of crossing the blood brain barrier and get into the brain, which means that it has no efficiency in LSD with central nervous system involvement, as is the case of MLD.

Substrate deprivation therapy is also under development for several LSD (Platt, *et al.*, 1997; Platt *et al.*, 2001; Abe *et al.*, 2000; Jeyakumar *et al.*, 1999), and more selective and potent inhibitors of glycosphingolipid synthesis are being investigated (Lee *et al.*, 1999; Abe *et al.*, 2000).

Since the inhibitors that have been used have the capacity of crossing the blood brain barrier, this seems to be a strategy whit great potential for LSD with central nervous system involvement. However, a major limitation results from the fact that these inhibitors act on the enzyme implicated in the glucosylceramide synthesis (Platt *et al.*, 1994) and thus they are only efficient in the LSD in which the accumulated substrate derives from this glycosphingolipid. So, in spite of its potential use in other LSD, this kind of therapy also does not look promising in the case of MLD.

Several observations indicated the bone marrow transplantation (BMT) as an important option for MLD therapy. As with lysosomal enzymes, ARSA can be secreted by one cell and internalised and delivered to the lysosomes of a neighbour cell through the mannose-6-phosphate receptors on the cell surface (Neufeld *et al.*, 1991). It has also been demonstrated that even very low amounts (5%) of endocytosed enzyme can be sufficient to correct the metabolic defect associated with MLD (Leinekugel *et al.*, 1992; Penzien *et al.*, 1993). The ARSA would have to be delivered to the glial cells in the brain, which are not accessible to plasma enzymes due to the blood-brain-barrier, but it had been shown that bone marrow derived monocytes/macrophages can cross the blood brain barrier and became perivascular macrophages and microglia (Krivit *et al.*, 1995; Krivit *et al.*, 1999). It thus seemed that normal monocytes of a donor bone marrow could originate in a MLD patient, resident microglial cells secreting normal ARSA and delivering it to the ARSA-deficient glial cells. With these observations, bone marrow

transplantation or bone marrow transplantation combined with gene therapy, were thus considered potentially useful therapeutic approaches for LSD with central nervous system manifestations, as is the case of MLD.

Nevertheless, it was recently reported that both macrophages and microglial cells secrete lysosomal enzymes that lack Man-6-P residues, which means that another mechanism, independent of Man-6-P-dependent secretion and recapture of lysosomal enzymes would have to be responsible for the therapeutic effects of BMT in brain (Muschol *et al.*, 2002).

Although several MLD patients had bone marrow transplantations, it is still not defined whether this is a valuable therapeutic option or not. The data available at the moment seem to indicate that in the case of symptomatic late infantile patients it is not effective, but regarding the cases of pre-symptomatic late infantile patients and late onset patients different results have been obtained. A follow-up of a significant number of transplanted patients has to be made before definitive conclusions can be reached (for reviewing see von Figura *et al.*, 2001).

Two different groups attempted to correct ARSA-deficient mice symptoms by gene therapy. Matzner *et al.* (2000, 2002), transplanted ARSA-deficient mice with genetically modified bone marrow cells overexpressing ARSA from a retroviral vector. The authors could observe a sulfatide storage reduction in liver but not in kidney and brain, this is in spite of the high enzyme activities in these tissues, resulting from a sustained high expression. Only a minor improvement in the neurological symptoms of treated mice was observed.

Another group (Consiglio *et al.*, 2001) reported the protection of ARSA-deficient mice from neuropathology using direct gene delivery into the central nervous

system by lentiviral vectors. Therefore, gene therapy remains a promise, but the experimental support for its efficiency is very limited and it can not be forgotten that the MLD mouse pathology only in part resembles human pathology, progressing more slowly and with less severity (Hess *et al.*, 1996b).

# GENERAL AIMS AND OUTLINE OF THIS THESIS

## GENERAL AIMS AND OUTLINE OF THIS THESIS

The general aims of this thesis were:

1 - To define the genetic epidemiology of MLD and ARSA-PD in Portugal.

2 - To study the impact of ARSA mutations in the structure, function and localization of the enzyme.

3 – To contribute to the establishment of a genotype/ phenotype correlation in MLD patients.

4 – To investigate the association of ARSA-PD with schizophrenia.

Before the beginning of this work, nothing was known regarding the genetic epidemiology of both ARSA deficiencies (MLD and ARSA-PD) in Portugal. To address this question we started by the definition of the mutation profile of the Portuguese MLD patients and by the analysis of the haplotype associated with each mutation. In this severe pathology, without an effective therapy, the pre-natal diagnosis and the identification of carriers within the families is very important since it allows the prevention of this disease. The molecular diagnosis is fundamental to the identification of heterozygotes and to exclude the existence of ARSA-PD, which represents a problem for the biochemical diagnosis of MLD based on the ARSA activity determination. The identification of the mutations associated with the different MLD clinical forms further contributes to the establishment of genotype-phenotype correlations that might be helpful to predict the age of onset and progression of the disease when associated with particular mutations. These results are presented in Chapter 1 - Section 1A.1 *(Marcão et al., Human Mutation (1999) 13(4): 337-8)* and Chapter 1 – Section 1A.2 *(Marcão et al., manuscript in preparation)*.

Three different alleles have been found to be associated with ARSA-PD, they can present very different frequencies in different populations and several hypotheses regarding their spatial and temporal origin remain speculative. Considering the interference of this condition in the MLD biochemical diagnosis and the possibility of its implication in the pathogenesis of some MLD non-related pathologies, we decided to determine the frequency and haplotype of the ARSA-PD associated alleles in the Portuguese population. These studies were important at a public health point of view and at a populations' genetics study point of view. The results are presented in Chapter 1 – Section 1B *(Marcão et al., Mol Genet Metab (2003) 79(4): 305-307).* As a first attempt to analyse the possibility of an increased risk of ARSA-PD individuals to develop schizophrenia, the frequency of the ARSA-PD alleles was also determined in a group of schizophrenic patients. The preliminary results on this subject are also presented in Chapter 1 – Section 1B.

Regardless of the three-dimensional structure of ARSA being resolved and the amino acids constituting the active site of the enzyme being identified, it is still not easy to predict for certain the effect of a particular alteration in the enzyme's structure and function. The identification of several non-conservative amino acid substitutions that do not lead to a decrease of ARSA activity, clearly illustrates the importance of doing expression analysis of every novel alteration found in the ARSA coding gene, especially in the case of missense mutations. These studies not only contribute to the elucidation of a genotype-phenotype correlation in MLD, but can also reveal some important amino acid residues or domains of the protein. The results of the expression of three novel mutations in the ARSA codifying gene are presented in Chapter 2 - Section 2A.1 *(Marcão et al., Am J Med Genet (2003) 116A(3): 238-42)* and Chapter 2 – Section 2A.2

*(the results presented in this section of Chapter 2 are part of a paper resulting from a collaboration with two other groups and that is submitted for publication).*

In the case of multimeric lysosomal enzymes like ARSA, the reason for the low in vivo activity associated with a specific mutation is not always easy to identify. The residual specific activity of the mutated enzyme is not the only important parameter. The enzyme has to be activated in the ER through a posttranslational modification common to all sulfatases, it has to be correctly folded, in order to escape to the ER quality control machinery, and it has to be able to reach the Golgi. In the Golgi, it has to be correctly phosphorylated, in order to be recognized by the Man-6-P receptors and to be correctly targeted to the lysosomes. Once in the lysosome, the enzyme has yet to be able to octamerize, otherwise it is rapidly degraded by the lysosomal proteases. The biochemical characterization of each mutation, including the analysis of these different steps, can thus contribute to a more general understanding of the normal cellular processes of posttranslational modification, folding, traffic through the cell, targeting to the lysosome, stabilization and activity in the lysosome of the lysosomal soluble enzymes. Additionally, these studies contribute to the understanding of the general picture of the cell functioning in pathological situations, which is essential for the development of new therapeutic strategies for lysosomal storage disorders. In this perspective, we studied the effect of three novel missense mutations on the stability and correct targeting of ARSA to the lysosome (Chapter 2 – Section 2A) and the effect of two of these mutations on the quaternary structure of ARSA (Chapter 2 – Section 2B, *Marcão et al., Biochem Biophys Res Commun (2003) 306(1): 293-7*).

# CHAPTER 1. GENETIC EPIDEMIOLOGY OF ARSA DEFICIENCIES IN THE PORTUGUESE POPULATION

## 1A. MLD IN PORTUGAL

### 1A.1. IDENTIFICATION OF NEW MOLECULAR LESIONS

### 1A.2. MUTATION PROFILE OF MLD PATIENTS

## 1B. ARSA-PD ASSOCIATED ALLELES IN THE PORTUGUESE POPULATION

# 1A. MLD IN PORTUGAL

# 1A.1. IDENTIFICATION OF NEW MOLECULAR LESIONS

*MUTATION IN BRIEF*

# Metachromatic Leucodystrophy in Portugal—Finding of Four New Molecular Lesions: C300F, P425T, g.1190-1191insC, and g.2408delC

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ABSTRACT

**The mutation identification rate achieved in the study of Portuguese MLD patients was found to be extremely high (100%), thus revealing the power of the association of vertical and horizontal PCR-SSCA. The identification of new mutations adds to the large number of mutations already described to be associated to MLD. Nevertheless, mutation g.1238G>A has been found in most of the Portuguese patients, either in homozygosity or heterozygosity, suggesting this mutation to be more common in Portuguese patients than in patients with other ethnic backgrounds. Two new missense mutations (C300F and P425T) were found to be associated to late infantile and juvenile forms, respectively. Two novel microlesions (g.1190-1191insC, g.2408delC) were identified in two late infantile patients. It should be noted that both C300F and g.2408delC were detected in homozygosity.**

**The approach used and the results here presented may provide useful information for the study of other MLD patients, as well as new insights about the effect of mutations, such as C300F, in the structure/function of ARSA.** © 1999 Wiley-Liss, Inc.

KEY WORDS: Metachromatic leucodystrophy; Portuguese population; ARSA

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## INTRODUCTION

Metachromatic Leucodystrophy (MLD, MIM# 250100) is an autosomic recessive disorder (estimated frequency of 1:100,000) usually caused by deficient arylsulfatase A (ARSA, EC 3.1.6.8) and by the intralysosomal accumulation of its substrate, cerebroside sulfate. Three major clinical forms are generally considered according to the age of onset (late infantile, juvenile and adult forms). The late infantile form is the most frequent clinical form of MLD, and is characterized by onset between ages of fifteen months and two years of age (Kolodny and Fluharty, 1995).

Located on chromosome 22q13, the ARSA gene spans 3.2 kb (with a 1521 bp cDNA), comprises 8 exons (Kreysing et al., 1990) and encodes a 507 aa protein (Stein et al., 1989). Of the numerous point mutations described in the ARSA gene, very few ARSA mutations are public. The two most common ARSA mutations are mutation g.1238G>A (designated as allele "I", MIM# 250100.0003) and mutation P426L (also called allele "A", MIM# 250100.0004). Both of these mutations are known to account for about 50% of all MLD causing alleles (Polten et al., 1991).

Despite the high frequency of allele g.1238G>A (79%) among unrelated Portuguese MLD patients (Amaral et al., 1998) some heterogeneity has been found. Extensive scanning of the ARSA exons and flanking regions, by SSCA allowed full characterization of the Portuguese MLD patients and resulted in the detection of four new molecular lesions, two in homozygosity and two in compound heterozygosity. Since the lowest rate of MLD allele detection seems to be among late infantile patients (Berger et al., 1997), the approach pertaining to the identification of these novel mutations associated with this form of the disease, may provide new clues for the molecular analysis of other European MLD populations.

## MATERIAL AND METHODS

### Patient description

The patients presented in this study originate from various regions of Portugal. All three late infantile patients presented onset between 12 and 24 months of age with death arising before seven years of age. With regard to the juvenile patient, symptoms were detected at age five and death occurred at thirteen years of age. Two of the cases here presented (patients C and D), were only clinically and biochemically diagnosed, no biological materials were available at the time of the molecular characterization. In both cases the molecular studies were performed on the biological material from both parents. Whenever possible the patients' parents were also studied in order to corroborate the patient results. In all cases, samples were obtained with informed consent.

### Population studied in the screening of the novel mutations

Blood samples from fifty healthy individuals were randomly collected among blood bank donors.

### Biochemical studies

Patients were biochemically diagnosed by ARSA activity determination in leucocytes (Baum et al., 1959; Lee-Vaupel and Conzelmann, 1987) and by the study of sulfatide excretion in urine (unpublished method).

### Molecular Studies

Genomic DNA was extracted from frozen leucocyte pellets, fibroblasts or buffy coats following standard methods.

Patients, or their parents when biological material from the patients was not available, were first screened for the presence of the two most frequent MLD causal mutations by Mva I or by ASOH (in the case of mutation g.1238G>A and P426L, respectively). The PCR conditions used for the amplification of various regions of the ARSA gene were as indicated in table 1. The ARSA haplotype was also determined (Zlotogora et al. 1994).

The application of both horizontal (Amaral et al., 1996) and vertical (Ribeiro et al., 1996) SSCA resulted in the identification of 100% of the patients' alleles. Samples presenting abnormal migration patterns were submitted to direct sequencing of asymmetric PCR generated products. In all cases different PCR samples were sequenced to confirm the reproducibility of the results and when possible the alteration was confirmed by restriction endonuclease digestion. Whenever possible the findings were corroborated by the analysis of the

patients' parents. In the two cases in which no biological material was available from the patients, the parents were exhaustively tested by SSCA.

Most reagents were from Boehringer Mannheim (GmbH, W. Germany), Pharmacia Biotech (Upsalla, Sweden), PE Applied Biosystems or from Sigma (Spain).

## RESULTS

As shown in table 2, patients A and D were compound heterozygotes for g.1238G>A and P426L (late infantile and juvenile patients, respectively), whereas patients B and C did not present either mutation.

Examination by PCR-SSCA and sequencing of samples presenting abnormal migration patterns resulted in the detection of four new mutations (table 2).

The g.3006C>A transversion (P425T) was confirmed by restriction endonuclease Bmy I digestion. In the case of the C300F mutation (patient C) a different approach was followed, a mismatched primer was designed in order to create a site for the restriction endonuclease Sfu I, in the presence of this mutation (table 1).

The screening of mutations C300F (Sfu I) and P425T (Bmy I), in a sample of 100 alleles indicated that these mutations were not polymorphic.

It would be interesting to further study the new mutations presented in this paper, unfortunately the lack of immortalized biological materials from most of these patients makes it difficult.

## DISCUSSION

### Late infantile patients

The identification of C300F in both parents of patient C indicated that this patient must have been homozygous for this novel mutation. The fact that C300 is conserved among murine and human ARSA (Kreysing et al., 1994) suggests that this residue is important for the structure and function of the protein. With the publication of ARSA's crystal structure it is possible to see that this mutation leads to the disruption of the disulfide covalent bond between amino acids 300 and 414, linking the two major and minor β-sheets (Lukatela et al., 1998). The finding of this novel mutation in the non-consanguineous parents of patient C indicated that this might be a rare but not private mutation.

The identification of a novel insertion (g.1190-1191insC) in exon 2 of patient A contributes to the large number of mutations already described in this exon. The resulting frameshift leads to the appearance of a stop codon in exon 3, this patient bears another null allele, allele g.1238G>A.

Patient B, homozygous for a novel microdeletion in exon 6 (g.2408delC), was the daughter of consanguineous parents (first cousins) thus suggesting a private mutation. This deletion leads to the appearance of a stop codon in exon 8, predicting the truncation of the protein about 84 aminoacids before its carboxi terminus. The identification of this nonsense mutation in homozygosity, in a late infantile patient, indicates that this is also a null allele.

### Juvenile Patient

Patient D was never studied at the molecular level since biological material was unavailable. The study of the parents revealed that the father carried allele P426L, while the mother carried mutation P425T.This seems to be a novel and causal mutation, this proline is preserved in the murine ARSA gene (Kreysing et al.,1994), in the sea urchin and sulfatases A, B and C (Kolodny and Fluharty, 1995). In this case, and according to the ARSA crystal structure, the regulation of dimer octamer association is probably impaired and must result in rather detrimental effects at the enzyme level.

## SUMMARY

In this work we report the identification of four new highly deleterious ARSA mutations found in Portuguese MLD patients: two microlesions (in late infantile patients) and two missense lesions (in a homozygous late infantile patient and in a juvenile patient with one low residual activity allele).



## ACKOWLEDGMENTS

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## REFERENCES


Amaral O, Pinto E, Fortuna M, Lacerda L, Sá Miranda MC.1996. Type 1 Gaucher Disease: Identification of N396T and Prevalence of Glucocerebrosidase Mutations in the Portuguese. Hum Mutat 8:280-281.

Amaral O, Marcão M, Pinto E, Sá Miranda MC.1998. Mutation analyses of the three most common lysosomal storage disorders in Portugal. Eur J Hum Genet 6 (sup.1): P1.027.

Baum H, Dogsan KS, Spencer B.1959. The assay of arylsulfatase A and B in urine. Clin Chim Acta 4:453-455.

Berger J, Loschl B, Bernheimer H, Lugowska A, Tylki-Szymanska A, Gieselmann V, Molzer B. 1997.Occurrence, Distribution, and Phenotype of Arylsulfatase A Mutations in Patients With metachromatic leukodystrophy. Am J Med Genet 69:335-340.

Kolodny EH, Fluharty AL. 1995. Metachromatic Leukodystrophy and Multiple Sulfatase Deficiency: Sulfatide Lipidoses. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds): The metabolic basis of inherited disease. New York: McGraw-Hill, p 2671-2739.

Kreysing J, von Figura K, Gieselmann V. 1990. Structure of the arylsulfatase A gene. Eur J Biochem 191:627-631.

Kreysing J, Polten A, Hess B, von Figura K, Menz K, Steiner F, Gieselmann V. 1994. Structure of the Mouse Arylsulfatase A Gene and cDNA. Genomics 19:249-256.

Lee-Vaupel M, Conzelmann E. 1987. A simple chromogenic assay for Arylsulfatase A. Clin Chim Acta 164(2): 171-180.

Lukatela G, Krauss N, Theis K, Selmer T, Gieselmann V, von Figura K, Saenger W. 1998. Crystal structure of human arylsulfatase A: the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis. Biochemistry 37(11): 3654-3664.

Ribeiro MG, Sonin T, Pinto RA, Fontes A, Ribeiro H, Pinto E, Palmeira MM, Sá Miranda MC. 1996. Clinical, enzymatic, and molecular characterisation of a Portuguese family with a chronic form of GM2-gangliosidosis B1 variant. J Med Genet 33:341-343.

Polten A, Fluharty AL, Fluharty CB, Kappler J, von Figura K, Gieselmann V. 1991. Molecular basis of different forms of metachromatic leukodystrophy. New Eng J Med 324:18-22.

Stein C, Gieselmann V, Kreysing J, Schmidt B, Pohlmann R, Waheed A, Meyer HE, O'Brien JS, von Figura K. 1989. Cloning and Expression of Human Arylsulfatase A. J Biol Chem 264(2): 1252-1259.

Zlotogora J, Furman-Shaharabani Y, Goldenfum S, Winchester B, von Figura K, Gieselmann V (1994) Arylsulfatase A pseudodeficiency: A common polymorphism which is associated with a unique haplotype. Am J Med Genet 52:146-150.

Table 1: PCR primers and conditions for the amplification of different ARSA fragments.

| Exon included in the PCR fragment | Size of PCR product | Sequence of oligonucleotide primers (5'-3') | PCR conditions ( 30 cycles ) |
|---|---|---|---|
| Exon 1 | 328 bp | AGCCTGCTGGAGCCAAGTA<br>AGGAAAGGGATGGAGGGTC | 1′ - 94 °C<br>5′ - 66 °C |
| Exon 2 | 362 bp | TCAGGGACTCTGTGACTTGTC<br>TGAGGGCTGGGCTGGCAGGTG | 40′′ - 94 °C<br>2′30′′ - 66 °C |
| Exon 3 | 269 bp | CCCAGCCCTCACGGCAGCT<br>GTTGGGCCAAGATCACTTA | 40′′ - 94 °C<br>2′30′′ - 66 °C |
| Exon 4 | 323 bp | ACGTAAGTGATCTTGGCCC<br>GCAAGCAGCTGAACTGCAA | 40′′ - 94 °C<br>2′30′′ - 66 °C |
| Exon 5 | 235 bp | TCAGAGGCTGTGGCTCCAT<br>CAGTTCGCCATCAAGGTTG | 1′ - 94 °C<br>5′ - 66 °C |
| Exon 5 (3' end) | 116 bp | TCAGAGGCTGTGGCTCCAT<br>CGTAGGTCGTTCCCTTTCGA [a] | 35′′ - 94 °C<br>40′′ - 58 °C<br>45′′ - 72 °C |
| Exon 6 | 217 bp | CCTTGATGGCGAACTGAG<br>AGGACAGGGGCCAAGGAT | 1′ - 94 °C<br>5′ - 66 °C |
| Exon 7 | 291 bp | GGTTCCTGGGTGGGCAAGAAG<br>AGGGATCTAGGGCTCCGGGG | 40′′ - 94 °C<br>2′30′′ - 66 °C |
| Exon 8 (5' end) | 309 bp | CCTTGCCCTGTGCACAGAA<br>GCAGCCAGGATGACAGCAGAT | 40′′ - 94 °C<br>2′30′′ - 66 °C |
| Exon 8 (3' end) | 304 bp | CTGCTCAAGGCCCAGTTAG<br>ACCAGCACAGCATTACC | 40′′ - 94 °C<br>2′30′′ - 66 °C |

[a]The underlined nucleotide represents a mismatch in order to create a Sfu I site in the presence of the mutated sequence.

Table 2: Summarized characteristics of cases with novel mutations.

| Patient | Clinical form of MLD | ARSA Activity (nmol/h/mg) | Genotype | Novel Molecular lesion[c] | Detection method of new mutations | Altered exon | Haplotype[d] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Bgl I | Bam HI | Bsr I |
| A | Late infantile | 0[a] | g.1238G>A/ g.1190-1191insC | g.1190-1191insC (Frameshift) | Vertical SSCA /sequencing | 2 | (1), (2) | (1), (1) | (1), (2) |
| B | Late infantile | 0[a] | g.2408delC/ g.2408delC | g.2408delC (Frameshift) | Vertical SSCA /sequencing | 6 | (2), (2) | (1), (1) | (1), (1) |
| C* | Late infantile | 0[a] | C300F/C300F | g.2177G>T (Cys300Phe) | Horizontal SSCA /Sfu I digestion | 5 | (2), (2) | (1), (?) | (?), (?) |
| D* | Juvenile | 5[b] | P426L /P425T | g.3006C>A (Pro425Thr) | Horizontal SSCA /Bmy digestion | 8 | (2), (2) | (1), (?) | (?), (?) |

*Biological material from these patients was not available for the molecular studies. The study was done in the patients' parents DNA.

[a]ARSA activity determined according to Lee-Vaupel M and Conzelmann E, 1987 (reference values in our Department: 6-15 nmol/h/mg).

[b]ARSA activity determined according to Baum et al., 1959 (reference values in our Department : 43-234 nmol/h/mg).

[c]Genomic sequence numbering according to GenBank X52150.

[d]Haplotype designation according to Zlotogora et al., 1994.

# 1A.2. MUTATION PROFILE OF MLD PATIENTS

**MUTATION PROFILE OF METACHROMATIC LEUKODYSTROPHY IN PORTUGAL: COMPARISON WITH OTHER EUROPEAN MLD POPULATIONS**

Marcão A, Pinto E, Silva E, Rocha S, Pinto R, Amaral O and Sá Miranda MC

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**Abstract**

Metachromatic Leukodystrophy is an autosomal recessive disorder usually caused by deficient Arylsulfatase A (ARSA). In this work, sixteen unrelated Portuguese patients with deficient ARSA activity were investigated for molecular lesions in the ARSA coding gene. Mutation IVS2+1G>A was found in 60% of the MLD causal alleles, suggesting this mutation to be more common in Portuguese patients than in patients with other ethnic backgrounds. Its finding at such a high frequency and in linkage disequilibrium with its originally described haplotype, supports the idea of a common ancient European origin for this mutation. On the contrary, mutation P426L, reported as the most frequent MLD mutation among late onset patients, was found in only one allele, in one juvenile patient. Six additional molecular lesions were identified by an SSCA-sequencing strategy, thus confirming the molecular heterogeneity reported in MLD. The ARSA-PD associated alterations were not found in any of these patients. Two missense mutations previously described in non-Portuguese patients, I179S and D255H, were both found in heterozygosity with allele IVS2+1G>A and associated to adult and late infantile MLD, respectively. Two microlesions (c412insC, c1040delC) and one missense mutation (C300F) were found to be associated to the late infantile form, while another missense mutation (P425T) was found in a juvenile patient. All these four mutations were reported for the first time in Portuguese patients, and only

mutation c412insC was recently also reported in a Greek patient (Coulter-Mackie *et al.*, 2003). Some clinical heterogeneity was found among the adult patients having the same genotype, even within the same family. Moreover, the patients belonging to two of the three families with adult patients do not seem to excrete elevated amounts of sulfatide in urine, which also indicates some biochemical heterogeneity.

**Key Words:** Metachromatic Leukodystrophy; Portugal; Arylsulfatase A; mutations; haplotype.

## Introduction

Metachromatic Leukodystrophy (MLD, McKusick 250100) is an autosomic recessive disorder usually caused by deficient Arylsulfatase A (ARSA, EC 3.1.6.8) and characterised by the intralysosomal accumulation of its substrate, 3-O-sulfo-galactosylceramide (sulfatide). This leukodystrophy presents three major clinical forms (late infantile, juvenile and adult), classified according to the age of onset. Heterogeneity exists in the age of onset and clinical course of the disease, being particularly evident in the adult form (for review see von Figura *et al.*, 2001).

The ARSA locus is located in chromosome 22q13, the gene is divided into 8 exons spanning 3.2 Kb (Kreysing *et al.*, 1990). The cDNA is 1521 bp long and codifies a 507 amino acids protein (Stein *et al.*, 1989). Presently, over 90 mutations have been described in the ARSA gene as giving rise to decreased enzyme activity (von Figura *et al.*, 2001 and *www.hgmd.org*). The two most common ARSA mutations in European populations are mutation IVS2+1G>A (allele I) and mutation P426L (allele A), first described as associated with late infantile and adult clinical forms, respectively (Polten

*et al.*, 1991). Mutation I179S is the third most common mutation in Europe and has been always found associated with late onset clinical forms, even when in heterozygosity with null mutations (von Figura *et al.*, 2001). Most of other mutations identified in the ARSA coding gene were found in single families or in isolated ethnic groups.

Based on the linkage disequilibrium verified between mutation IVS2+1G>A and a single rare haplotype, defined by three intragenic polymorphisms, Zlotogora *et al.* (1994) suggested a common origin for all IVS2+1G>A alleles. This haplotype was later extended to other two intragenic polymorphisms by Coulter-Mackie *et al.* (1997).

In Portugal, MLD has a prevalence of approximately 1: 50 000 (Pinto *et al.*, in press).

In this **work** we present the results of the molecular study of twenty Portuguese MLD patients belonging to sixteen unrelated families. After testing IVS2+1G>A and P426L mutations, the application of an SSCA (Single Strand Conformation Analysis)/ Sequencing strategy allowed full characterisation of the patients with the detection of six additional molecular lesions. Haplotype analysis regarding five intragenic polymorphisms was also performed.

## Material and Methods

### *Patients*

A total of twenty patients belonging to sixteen unrelated families, and originating from various regions of Portugal, were included in this study. Several patients (I, K, M, N, O and P) were previously referred in other publications (Alves *et al.*, 1986; Marcão *et al.*, 1999). All the late infantile patients (12 unrelated patients)

presented a classical clinical evolution with onset occurring around two years of age and originated from various regions of Portugal. The juvenile patient (N) was from the northern region of Portugal. He started to develop symptoms at 5 years of age and died with 13 years of age. Adult patients from families 14 and 15 also originated from the north of Portugal, whereas the adult patient T was from Azores. We have no detailed clinical information regarding patients from family 15. Patients from families 14 and 16 first presented with behavioural disturbances, with normal neurological examinations and without any motor signs. Progressive intellectual deterioration developed in all of them.

***Material***

Oligonucleotides were synthesised by Amersham Pharmacia Biotech (Freiburg, Germany). Restriction endonucleases were from New England Biolabs (Frankfurt am Main, Germany) or Roche Applied Science (Mannheim, Germany). Taq DNA polymerase was from Invitrogen (Groningen, The Netherlands). NuSieve® 3:1 agarose was from BioWhittaker Molecular Applications (BMA, Rockland, USA). Other reagents were from Sigma (Sigma-Aldrich, St Louis, USA) or Merck (Darmstadt, Germany). PCR was performed in a Biometra-Uno II termocycler (Biometra, Göttingen, Germany). Sequencing reactions were prepared using the DNA Sequencing Kit BigDye$^{TM}$ Terminator Cycle sequencing Ready Reaction from Applied Biosystems and ran on the ABI PRISM$^{TM}$ 310 Genetic Analyser from Applied Biosystems (PE, Applied Biosystems, Foster City, CA).

***Methods***

***Biochemical studies***

Patients were biochemically diagnosed in the Enzymology Department of IGMJM, by ARSA activity determination in leucocytes and fibroblasts (Baum *et al.*, 1959; Lee-Vaupel and Conzelmann, 1987) and by the study of sulfatide excretion in urine (Berna *et al.*, 1999). The activity of ARSA was also analysed in leukocytes and fibroblasts, after separation of the different arylsulfatases by an electrophoresis performed on sheets of cellulose acetate gels (Alves *et al.*, 1986).

***Molecular Studies***

Genomic DNA was extracted from frozen leukocyte pellets, fibroblasts or buffy coats following standard methods.

*1. Screening for frequent alterations*

Mutations IVS2+1G>A and P426L were tested by Allele Specific Oligonucleotide Hybridization (Polten *et al.*, 1991) or by restriction analysis as indicated in Table 1.

The ARSA-PD associated alterations, A3352G and N350S, were tested by Mae III restriction analysis and allele-specific PCR, respectively (Marcão *et al.*, 2003a).

*2. Identification of other alterations*

Cases that remained with unknown mutations, after the initial screening for frequent alterations, were analysed by non-isotopic PCR-SSCA. The application of both horizontal (Amaral *et al.*, 1996) and vertical (Ribeiro *et al.*, 1996) SSCA analysis to nine fragments comprising all the ARSA exons (Table 1) resulted in the identification of 100% of the patients' alleles. Fragments presenting abnormal migration patterns were further examined by direct sequencing of asymmetric PCR generated products. In all

cases independent PCR samples were sequenced to confirm the reproducibility of the results.

**Table 1 - Amplification and analysis of different ARSA gene fragments**

| ARSA gene fragment [a] | Oligonucleotide sequence [b] | PCR ann. temp. (°C) | Type of analysis |
|---|---|---|---|
| ARSA571-898 | 5'AGCCTGCTGGAGCCAAGTA3' | 66 | SSCA/ sequencing |
| | 5'AGGAAAGGGATGGAGGGTC3' | | |
| ARSA970-1332 | 5'TCAGGGACTCTGTGACTTGTC3' | 66 | SSCA/ sequencing |
| | 5'TGAGGGCTGGGCTGGCAGGTG3' | | |
| ARSA1023-1571 | 5'CGGTTCGGATGGGCATGTAC3' | 66 | Mva I restriction analysis |
| | 5'CGTGAGAGGCATAGTACAGGAAGAA3' | | for IVS2+1G>A detection. |
| ARSA1322-1590 | 5'CCCAGCCCTCACGGCAGCT3' | 66 | SSCA/ sequencing |
| | 5'GTTGGGCCAAGATCACTTA3' | | |
| ARSA1569-1891 | 5'ACGTAAGTGATCTTGGCCC3' | 66 | SSCA/ sequencing |
| | 5'GCAAGCAGCTGAACTGCAA3' | | |
| ARSA2081-2314 | 5'TCAGAGGCTGTGGCTCCAT3' | 66 | SSCA/ sequencing |
| | 5'CAGTTCGCCATCAAGGTTG3' | | |
| ARSA2298-2513 | 5'CCTTGATGGCGAACTGAG3' | 66 | SSCA/ sequencing |
| | 5'AGGACAGGGGCCAAGGAT3' | | |
| ARSA2604-2887 | 5'GGTTCCTGGGTGGGCAAGAAG3' | 66 | SSCA/ sequencing |
| | 5'AGGGATCTAGGGCTCCGGGGA3' | | |
| ARSA2903-3212 | 5'CCTTGCCCTGTGCACAGAA3' | 66 | SSCA/ sequencing |
| | 5'GCAGCCAGGATGACAGCAGAT3' | | |
| ARSA2981-3136 | 5'CTCCAGCTCTCTGACTGCTCATCAG3' | 65 | Alw NI restriction analysis |
| | 5'GCGTCTAACTGGGCCTTGAGCAGC3' | | for P426L detection. |
| ARSA3114-3417 | 5'CTGCTCAAGGCCCAGTTAG3' | 66 | SSCA/ sequencing |
| | 5'ACCAGCACACAGCATTACC3' | | |

PCR reactions (100 µL): 1X PCR buffer, 1,5 mM $MgCl_2$, 0.2 mM dNTPs, 1mM primer #1, 1mM primer #2, 200-800 ng of gDNA, 0.02 U/ µL of Taq DNA polymerase. [a] Genomic DNA nucleotide numbering is according to Kreysing *et al.* (1990). [b] Mismatch nucleotides are underlined.

*3. Haplotype Studies*

W193C, g1923c, g2015a, T391S and c2842g polymorphisms were tested by endonuclease restriction digestion (Polten *et al.*, 1991; Coulter-Mackie *et al.*, 1997).

**Results**

Twelve of the sixteen non-related patients that were studied had mutation IVS2+1G>A, seven of them being homozygotes. Mutation P426L was only found in one juvenile patient, in heterozygosity with the missense mutation P425T (Table 2). None of the ARSA-PD associated alterations was found among the MLD patients.

Six additional alterations were detected by SSCA/ sequencing (Table 2). Two of these mutations, I179S (Fluharty *et al.*, 1991) and D255H (Lissens *et al.*, 1996), were previously described in non-Portuguese patients. The other four alterations were found in Portuguese families: two microlesions that consisted of a C insertion and a C deletion in positions 412 and 1040 of cDNA (Stein *et al.*, 1989), respectively, and two missense mutations that were a G899>T transversion causing substitution of Cys300 by Phe and a C1273>A transversion leading to the substitution of Pro425 by Thr (Marcão *et al.*, 1999). A summary of the results of the patients studied is presented in Table 2.

The patients homozygous for mutation IVS2+1G>A were also homozygous for each of the five polymorphisms examined (Table 3), thus presenting the fixed haplotype reported by Zlotogora *et al.* (1994). The results obtained for all the carriers of this mutation were also compatible with the existence of linkage disequilibrium with that same haplotype. Mutations c1040delC, D255H, I179S and c412insC were found in the context of different haplotypes as indicated in Table 3. In relation to mutations C300F, P426L and P425T (found in patients M and N, from whom no biological sample was

available at the time of the molecular characterisation), the associated haplotypes could only be partially determined by indirect study performed in the parents.

**Table 2- Molecular and biochemical analysis of Portuguese patients with MLD**

| Family | Patient | MLD clinical form | ARSA activity (nmol/h/mg) | Sulfatide in urine | Genotype |
|---|---|---|---|---|---|
| 1 | A | Late infantile | 0 [a] | Increased | I / I |
| 2 | B | Late infantile | 0 [a] | Nd | I / I |
| | C | Late infantile | 0 [a] | Increased | I / I |
| 3 | D | Late infantile | 0 [a] | Nd | I / I |
| 4 | E | Late infantile | 0 [a] | Increased | I / I |
| 5 | F | Late infantile | 0 [a] | Increased | I / I |
| 6 | G | Late infantile | 0[a] | Increased | I / I |
| 7 | H | Late infantile | 0[a] | Increased | I / I |
| 8 | I | Late infantile | 0[a] | Increased | c1040delC/ c1040delC |
| 9 | J | Late infantile | 0[a] | Increased | c1040delC/ c1040delC |
| 10 | K | Late infantile | 0[a] | Nd | I / c412insC |
| 11 | L | Late infantile | 0[a] | Increased | I / D255H |
| 12 | M* | Late infantile | 0[a] | Nd | C300F/C300F |
| 13 | N* | Juvenile | 5[b] | Nd | P426L /P425T |
| 14 | O | Adult | 0[a] | Increased | I / I179S |
| | P | Adult | 13[b] | Increased | I / I179S |
| | Q | Adult | 31[b] | Increased | I / I179S |
| 15 | R | Adult | 0[a] | Normal | I/ I179S |
| | S | Adult | 0[a] | Normal | I/ I179S |
| 16 | T | Adult | 0[a] | Normal | I/ I179S |

Nd - Not determined. *Biological material from these patients was not available for the molecular studies, the study was done in the patients' parents DNA. [a]ARSA activity determined in leukocytes according to Lee-Vaupel M and Conzelmann E (1987), (reference values in the Enzymology Department (IGMJM): 6-15 nmol/h/mg). [b]ARSA activity determined in leukocytes according to Baum *et al.* (1959), (reference values in the Enzymology Department (IGMJM): 43-234 nmol/h/mg). Families 8, 10, 12, 13 and 14 were referred in previous publications (Alves *et al.*, 1986; Marcão *et al.*, 1999).

**Table 3 – Haplotype analysis**

| | Polymorphisms | | | | |
|---|---|---|---|---|---|
| Restriction endonuclease | Bgl I | Bsr I | Bam HI | Nla III[2] | Nla III[1] |
| Mutations | W193C | T391S | c2842g | g1923c | g2015a |
| IVS2+1G>A | C | S | g | g | g |
| c412insC | W | T | g | a | g |
| I179S | W | T | c | a | g |
| D255H | W | S | g | g | g |
| C300F | W | S | g | nc | g |
| c1040delC | W | S | g | g | g |
| P425T | W | T | nc | nc | g |
| P426L | W | nc | g | g | g |

nc – non conclusive. The results for mutations I179S, D255H and c412insC assume the haplotype Bgl I (C ), Bsr I (S), Bam HI (g), Nla III[2] (g), Nla III[1] (g) for allele Ivs2+1G>A. Nla III[1] and Nla III[2] polymorphic sites denomination is according to Coulter-Mackie *et al.* (1997).

## Discussion

The mutation profile of Portuguese MLD patients seems to be different from what has been described for other European populations (Figure 1). The most striking differences are the higher frequency of an ARSA null mutation (IVS2+1G>A), which represents 60% of the MLD alleles among unrelated Portuguese patients (67% among the late infantile patients) and the lower frequency of mutation P426L, which represents only 3% of those alleles. This latter mutation was not found among eighteen unrelated Spanish MLD patients and thus also seems to have a low frequency in Spain.

Nevertheless mutation IVS2+1G>A represents only 25% of the MLD alleles in the Spanish population (Gort *et al.*, 1999).

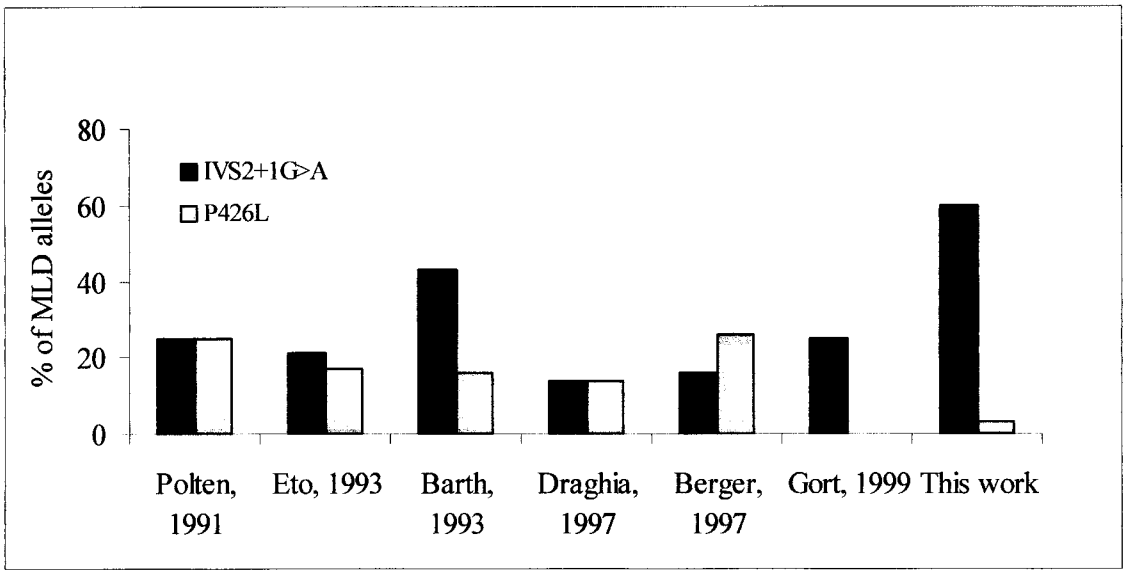


**Figure 1. Frequency of mutations IVS2+1G>A and P426L in different European MLD populations.**

Zlotogora *et al.* (1994) identified this mutation in both Christian and Arab families from Jerusalem and, based on its linkage desequilibrium with a single rare haplotype, proposed that it could have had an European ancient origin, being introduced in Jerusalem at the time of the Crusades. The fact that this mutation was found in different regions of Portugal, in such a high frequency and in linkage desequilibrium with the same rare haplotype, might indicate that this is an ancient mutation in our population and that the Portuguese could even have had contributed to its spread among other populations.

Haplotyping has also been done for the other mutant alleles and our results were in agreement with the fixed haplotypes previously proposed for mutations P426L, I179S

and D255H (Coulter-Mackie *et al.*, 1997). In spite of the small number of unrelated cases reported, these results suggest the possible existence of common ancestors.

As can be seen in Table 2, most late infantile patients (patients A to H) were homozygous for the null mutation IVS2+1G>A (Polten *et al.*, 1991). Three other patients were found to be homozygous for mutations c1040delC (patients I and J) and C300F (patient M), and two patients were found to be compound heterozygotes for mutation IVS2+1G>A and mutations c412insC (patient K) or D255H (patient L).

Mutation D255H, a null mutation first described in two Belgian late infantile patients, also compound heterozygotes with mutation IVS2+1G>A (Lissens *et al.*, 1996), seems to be relatively frequent in Spain where it represents 19% of the MLD alleles (Gort *et al.*, 1999). However, among the Portuguese MLD patients it was found in only one allele.

Mutation C300F was never found in a patient other than the one referred in Table 2. It originates an unstable, almost inactive enzyme with severe octamerization impairment in the lysosome (Marcão *et al.*, 2003b; Marcão *et al.*, 2003c). As expected from this severe biochemical phenotype, this mutation was found to be associated with the most severe clinical form of the disease (patient M).

Mutation c412insC, found in exon 2 of patient K is also likely to be null, as it results in frameshift with altered sequence after amino acid 138, leading to the appearance of a stop codon 36 amino acids later. This mutation was recently reported in an heterozygous late infantile patient of Greek origin (Coulter-Mackie *et al.*, 2003).

Patients I and J were both homozygous for a microdeletion in exon 6 (c1040delC). This deletion leads to the appearance of a stop codon in exon 8, predicting the truncation of the protein about 84 amino acids before its carboxy terminus.

Therefore, this is most certainly a null mutation, which is further supported by its identification in homozygosity in two late infantile patients.

Mutation c1040delC was found to be associated to the same haplotype in the two families in which it was identified. This fact may suggest the existence of a common ancestor, but the occurrence of this mutation in a repetitive sequence of the arylsulfatase A gene and its association to an haplotype that is common among the general population does not allow the exclusion of the possibility of an independent occurrence in both families.

The juvenile patient N was found to be a compound heterozygote for missense mutations P425T and P426L. Both these mutations originate ARSA proteins with some residual enzyme activity but that are unstable in the lysosome (Polten *et al.*, 1991; Marcão *et al.*, 2003b). Interestingly, both these mutations were also found to affect the enzyme octamerization capacity, thus explaining its instability (von Bülow *et al.*, 2002; Marcão *et al.*, 2003c).

All the adult patients, belonging to three different families, presented the same genotype (IVS2+1G>A/ I179S). Mutation I179S seems to be the third most common mutation among European MLD patients, presenting a frequency of about 19% (von Figura *et al.*, 2001). Even when in heterozygosity with null alleles, as is the case of the families here presented, this mutation has always been found associated with late-onset forms of MLD (von Figura *et al.*, 2001). In a recent publication, Baumann *et al.* (2002) reported its association with a more specific psycho-cognitive form of late onset MLD, characterized by initial behavioural disturbances and progressive mental deterioration. The same kind of phenotype was observed in the adult patients from families 14 and 16 (we have no clinical information regarding patients from family 15). In spite of this

apparent clinical homogeneity, some variability was observed in terms of age of onset and rate of progression, even among the various affected siblings reported here. Biochemical variability was also observed among these three Portuguese families, as patients from families 15 and 16 do not excrete elevated amounts of sulfatides in urine. This lack of elevated amounts of sulfatide in urine seems to be rare, but was also reported for an Italian juvenile patient (Gomez-Lira *et al.*, 1998) with the same genotype of the Portuguese patients (IVS2+1G>A/ I179S). The I179S mutation, which produces a mutant protein with some enzyme residual activity, but that is known to be particularly susceptible to proteolytic degradation (Fluharty *et al.*, 1991) may be in the origin of the variability encountered. In fact, juvenile and adult patients, with onset ages varying from 9 (Fluharty *et al.*, 1991) to 62 years of age (Perusi *et al.*, 1999), being compound heterozygotes for the I179S mutation and null alleles or alleles with some residual enzyme activity, have been reported. No clear correlation seems to exist between the biochemical phenotype of the 2$^{nd}$ mutation and the clinical phenotype. This heterogeneity raises the question of the existence of additional factors interfering with the "in vivo" activity of ARSA, and thus with the clinical expression of MLD.

In conclusion, a very high frequency of mutation IVS2+1G>A was found among the Portuguese MLD patients. Haplotype analysis corroborated the hypothesis that this mutation may have its origin in a common European ancestor. The molecular heterogeneity reported to be present in MLD patients is illustrated by the finding of eight different mutations among this group of sixteen non-related patients, three of which were never reported in non-Portuguese patients. Nevertheless, seven of the twelve late infantile patients were homozygous for mutation IVS2+1G>A and all adult patients have the same genotype (IVS2+1G>A/ I179S). Our results also confirm the

existence of a general genotype–phenotype correlation in MLD: late infantile patients have two null alleles, while the presence of at least one allele coding one enzyme with some residual activity mitigates the course of the disease originating a late onset form. However, some biochemical and clinical variability have been found among late onset patients. This is quite obvious from the comparison of the three adult families (all presenting the same genotype) and could be found even within each family. Given the fact that in the less severe forms of the disease the life expectancy of the patients is much longer, it seems likely that the effect of other environmental or genetic factors might become more evident, leading to a wide clinical heterogeneity, inter and intrafamilial, associated to the same arylsulfatase A genotype.

## References


Alves D, Pires M, Guimarães A, Miranda MC. Four cases of late onset metachromatic leukodystrophy in a family: clinical, biochemical and neuropathological studies. J Neurol Neurosurg Psychiatry (1986) 49: 1417-1422.

Amaral O, Pinto E, Fortuna M, Lacerda L, Sá Miranda MC. Type 1 Gaucher Disease: Identification of N396T and Prevalence of Glucocerebrosidase Mutations in the Portuguese. Hum Mutat (1996) 8: 280-281.

Barth ML, Fensom A, Harris A. Prevalence of common mutations in the arylsulphatase A gene in metachromatic leukodystrophy patients diagnosed in Britain. Hum Genet (1993) 91(1): 73-7.

Baum H, Dogsan KS, Spencer B. The assay of arylsulfatase A and B in urine. Clin Chim Acta (1959) 4: 453-455.

Baumann N, Turpin JC, Lefevre M, Colsch B. Motor and psycho-cognitive clinical types in adult metachromatic leukodystrophy: genotype/phenotype relationships? J Physiol Paris (2002) 96(3-4): 301-6.

Berna L, Asfaw B, Conzelmann E, Cerny B, Ledvinova J. Determination of urinary sulfatides and other lipids by combination of reversed-phase and thin-layer chromatographies. Anal Biochem (1999) 269(2): 304-11.

Berger J, Loschl B, Bernheimer H, Lugowska A, Tylki-Szymanska A, Gieselmann V, Molzer B. Occurrence, Distribution, and Phenotype of Arylsulfatase A Mutations in Patients With Metachromatic Leukodystrophy. Am J Med Genet (1997) 69: 335-340.

Coulter-Mackie M, Gagnier L. Two New Polymorphisms in the Arylsulfatase A Gene and Their Haplotype Associations With Normal, Metachromatic Leukodystrophy and Pseudodeficiency Alleles. Am J Med Genet (1997) 73: 32-35.

Coulter-Mackie MB, Gagnier L. Spectrum of mutations in the arylsulfatase A gene in a Canadian DNA collection including two novel frameshift mutations, a new missense mutation (C488R) and an MLD mutation (R84Q) in cis with a pseudodeficiency allele. Mol Genet Metab (2003) 79(2): 91-8.

Draghia R, Letourneur F, Drugan C, Manicom J, Blanchot C, Kahn A, Poenaru L, Caillaud C. Metachromatic Leukodystrophy: Identification of the First Deletion in Exon 1 and Nine Novel Point Mutations in the Arylsulfatase A Gene. Hum Mutat (1997) 9: 234-242.

Eto Y, Kawame H, Hasegawa Y, Ohashi T, Ida H, Tokoro T. Molecular characteristics in Japanese patients with lipidosis: Novel mutations in metachromatic leukodystrophy and Gaucher disease. Mol Cell Biochem (1993) 119(1-2): 179-84.

Fluharty AL, Fluharty CB, Bohne W, Von Figura K, Gieselmann V. Two new arylsulfatase A (ARSA) mutations in a juvenile metachromatic leukodystrophy (MLD) patient. Am J Hum Genet (1991) 49: 1340-1350.

Gomez-Lira M, Perusi C, Mottes M, Pignatti PF, Manfredi M, Rizzuto N, Salviati A. Molecular genetic characterization of two metachromatic leukodystrophy patients who carry the T799G mutation and show different phenotypes; description of a novel null-type mutation. Hum Genet (1998) 102(4): 459-63.

Gort L, Coll MJ, Chabas A. Identification of 12 novel mutations and two new polymorphisms in the arylsulfatase A gene: haplotype and genotype-phenotype correlation studies in Spanish metachromatic leukodystrophy patients. Hum Mutat (1999) 14(3): 240-8.

Kreysing J, von Figura K, Gieselmann V. Structure of the arylsulfatase A gene. Eur J Biochem (1990) 191: 627-631.

Lee-Vaupel M, Conzelmann E. A simple chromogenic assay for Arylsulfatase A. Clin Chim Acta (1987) 164(2): 171-180.

Lissens W, Vervoort R, van Regemorter N, van Bogaert P, Freund M, Verellen-Dumoulin C, Seneca S, Liebaers I. A D255H substitution in the arylsulphatase A gene of two unrelated Belgian patients with late-infantile metachromatic leukodystrophy. J Inher Metab Dis (1996) 19: 782-786.

Marcao A, Amaral O, Pinto E, Pinto R, Sá Miranda MC. Metachromatic leucodystrophy in Portugal-finding of four new molecular lesions: C300F, P425T, g.1190-1191insC, and g.2408delC, (Mutations in brief no. 232. Online.) Hum Mutat (1999) 13: 337-8.

Marcao A, Pinto E, Rocha S, Sa Miranda MC, Ferreira L, Amaral. ARSA-PD associated alleles in the Portuguese population: frequency determination and haplotype analysis. Mol Genet Metab (2003a) 79(4): 305-307.

Marcao A, Simonis H, Schestag F, Sá Miranda MC, Gieselmann V. Biochemical characterization of two (C300F, P425T) arylsulfatase a missense mutations. Am J Med Genet 116 (2003b) 238-42.

Marcao A, Azevedo JE, Gieselmann V, Sa Miranda MC. Oligomerization capacity of two arylsulfatase A mutants: C300F and P425T. Biochem Biophys Res Commun (2003c) 306(1): 293-7.

Pinto R, Caseiro C, Lemos M, Lopes L, Fontes A, Ribeiro H, Pinto E, Silva E, Rocha S, Marcão A, Ribeiro I, Lacerda L, Ribeiro G, Amaral O, Sá Miranda MC. The prevalence of lysosomal storage diseases in Portugal (in press, European Journal of Human Genetics).

Perusi C, Lira MG, Duyff RF, Weinstein HC, Pignatti PF, Rizzuto N, Salviati A. Mutations associated with very late-onset metachromatic leukodystrophy. Clin Genet (1999) 55(2): 130.

Polten A, Fluharty AL, Fluharty CB, Kappler J, von Figura K, Gieselmann V. Molecular basis of different forms of metachromatic leukodystrophy. New Eng J Med (1991) 324: 18-22.

Ribeiro MG, Sonin T, Pinto RA, Fontes A, Ribeiro H, Pinto E, Palmeira MM, Sá Miranda MC. Clinical, enzymatic, and molecular characterisation of a Portuguese family with a chronic form of GM2-gangliosidosis B1 variant. J Med Genet (1996) 33: 341-343.

Stein C, Gieselmann V, Kreysing J, Schmidt B, Pohlmann R, Waheed A, Meyer HE, O'Brien JS, von Figura K. Cloning and Expression of Human Arylsulfatase A. J Biol Chem (1989) 264(2): 1252-1259.

von Bülow R, Schmidt B, Dierks T, Schwabauer N, Schilling K, Weber E, Usón I, von Figura K. Defective oligomerization of arylsulfatase A as a cause of its instability in lysosomes and metachromatic leukodystrophy. J Biol Chem (2002) 277: 9455-9461.

von Figura K, Gieselmann V, Jaeken J. Metachromatic leukodystrophy. In: Scriver CR, et al, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill, 2001:3695-3724.

Zlotogora J, Furman-Shaharabani Y, Harris A, Barth ML, von Figura K, Gieselmann V. A single origin for the most frequent mutation causing late infantile metachromatic leucodystrophy. J Med Genet (1994) 31: 672-674.

# 1B. ARSA-PD ASSOCIATED ALLELES IN THE PORTUGUESE POPULATION

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Brief communication

# ARSA-PD associated alleles in the Portuguese population: frequency determination and haplotype analysis

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**Abstract**

Arylsulfatase A pseudodeficiency (ARSA-PD) may be related to increased susceptibility to neuro-psychiatric disorders. An association of allele 2417G/3352A with schizophrenia was found in a group of Portuguese patients. In the Portuguese population, at least one PD associated alteration exists in 18.3% of the ARSA alleles. Allele 2417G/3352G was invariably associated with a conserved haplotype, while 2417G/3352A and the rare 2417A/3352G alleles appeared on different haplotypes.
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*Keywords:* Arylsulfatase A pseudodeficiency; Metachromatic leukodystrophy; Schizophrenia

## Introduction

Metachromatic leukodystrophy (MLD) is an autosomal recessive disorder characterized by a severe central and peripheral nervous system involvement, and usually due to the deficiency in the lysosomal enzyme arylsulfatase A (ARSA, EC 3.1.6.8) [1]. Arylsulfatase A-pseudodeficiency (ARSA-PD), is a relatively frequent condition among the general population, which is characterized by a reduction to 5–15% of ARSA normal activity, and occurs in individuals who have no MLD and no sulfatide accumulation in tissues or excretion in urine [1]. The molecular defects in the origin of ARSA-PD consist of two A→G transitions in the ARSA coding gene (nucleotides 2417 and 3352 according to the GeneBank sequence X52150), that can be found isolated or combined, the first altering a N-glycosylation site and the second altering a polyadenylation signal [2].

The association between ARSA-PD and different neuro-psychiatric symptoms has been suggested by different authors (see [1] for review). In order to investigate the possible association of low ARSA activity and schizophrenia, we determined the frequency of ARSA-PD alleles in the Portuguese population and in a group of Portuguese patients suffering from schizophrenia.

The existence of several polymorphic sites within the ARSA gene, present in linkage disequilibrium in ARSA-PD alleles, has long been known [3]. The determination of ARSA-PD allele frequencies and haplotype analysis in different populations has raised several hypotheses regarding the order of appearance of the alterations that characterise the ARSA-PD alleles and its appearance as a single or a recurrent event [4]. Haplotype analysis of ARSA-PD and normal alleles, regarding six intragenic polymorphisms, was also carried out in this work.

## Materials and methods

### *Patients and control samples*

ARSA-PD associated alterations were studied in 161 healthy Portuguese blood donors (age ⩾ 18 years) and 33 Portuguese patients suffering from schizophrenia, according to the DSM-IV diagnostic criteria, and with a

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mean age of 47 years. Additionally, 23 individuals non-carriers of the ARSA-PD associated alterations, 10 individuals homozygous for allele 2417G/3352G, 12 individuals homozygous for allele 2417G/3352A and one heterozygote for both alleles, were used for the haplotype analysis. All blood samples were taken after informed consent was granted. Genomic DNA was isolated following standard methods.

*Material*

Oligonucleotides were provided by Amersham Pharmacia Biotech (Freiburg, Germany). Restriction endonucleases were from New England Biolabs (Frankfurt am Main, Germany) or Roche Applied Science (Mannheim, Germany). *Taq* DNA polymerase was from Invitrogen (Groningen, The Netherlands). Other reagents were from BioWhittaker Molecular Applications (BMA, Rockland, USA), Invitrogen (Groningen, The Netherlands) or Applied Biosystems (PE, Applied Biosystems, Foster City, CA).

*Methods*

The alterations associated with ARSA-PD (A2417G, A3352G), were tested as indicated in Table 1. To perform the haplotype analysis, four fragments, comprising the gene regions of six polymorphisms described in the ARSA codifying gene (G1471T, G1923C, A2015G, C2790G, C2842G, G3220A, nucleotide numbering according with GeneBank sequence X52150), were amplified by PCR and tested by restriction endonuclease analysis (Table 1).

**Results and discussion**

In the Portuguese control population, the two most frequent ARSA-PD alleles have approximately the same frequency (9.3 and 8.7%, respectively for alleles 2417G/3352G and 2417G/3352A), whereas the rare allele 2417A/3352G was found in only one individual, in heterozygosity with the allele 2417G/3352G. Taking the frequencies of the three alleles together, we have the highest value reported in a population of European origin (18.3%), which indicates the importance of ARSA-PD in the Portuguese population.

Seventeen different haplotypes were identified among the individuals for whom haplotype analysis was carried out. Looking at the percentage of each polymorphism in the control population we found *Bgl*I (G) in 97.8% of the alleles, *Bsr*I (G) in 61%, *Bam*HI (G) in 85%, *Nla*III$^2$(G) in 84.8%, *Nla*III$^1$ (G) in 93.5%, and *Aci*I (G) in 97.8% of the alleles.

The doubly mutated alleles 2417G/3352G were found to have exclusively one of the rare haplotypes: *Bgl*I (T) *Bsr*I (C) *Bam*HI (G) *Nla*III$^2$ (G) *Nla*III$^1$ (G) *Aci*I (G), which was found in 8.6% of the normal alleles and in none of the 2417G/3352A alleles. These results confirm the association of the doubly mutated ARSA-PD allele with a highly conserved haplotype [3], which together with its high frequency in Europeans and its absence in

Table 1
Detection of alterations in the ARSA gene by restriction endonuclease analysis and allele specific PCR

| Polymorphism | Reference | ARSA gene fragment | Oligonucleotide sequences | PCR ann. temp. | Detection method |
|---|---|---|---|---|---|
| G1471T (W193C) | Polten et al., N. Engl. J. Med., 1991 | ARSA1322/ ARSA1590 | 5′CCCAGCCCTCACGGCAGCT3′ 5′GTTGGGCCAAGATCACTTA3′ | 66 | *Bgl*I |
| G1923C (intron 4) | Coulter-Mackie et al., Am. J. Med. Genet., 1997 | ARSA1569/ ARSA2058 | 5′ACGTAAGTGATCTTGGCCC3′ 5′CCCACACCTCTAAGTCACA3′ | 58 | *Nla*III |
| A2015G (intron 4) | Coulter-Mackie et al., Am. J. Med. Genet., 1997 | ARSA1569/ ARSA2058 | 5′ACGTAAGTGATCTTGGCCC3′ 5′CCCACACCTCTAAGTCACA3′ | 58 | *Nla*III |
| C2790G (T391S) | Polten et al., N. Engl. J. Med., 1991 | ARSA2604/ ARSA2887 | 5′GGTTCCTGGGTGGGCAAGAAG3′ 5′AGGGATCTAGGGCTCCGGGGA3′ | 66 | *Bsr*I |
| C2842G (intron 7) | Polten et al., N. Engl. J. Med., 1991 | ARSA2604/ ARSA2887 | 5′GGTTCCTGGGTGGGCAAGAAG3′ 5′AGGGATCTAGGGCTCCGGGGA3′ | 66 | *Bam*HI |
| G3220A (R496H) | Ricketts et al., Hum. Mutat., 1998 | ARSA3114/ ARSA3417 | 5′CTGCTCAAGGCCCAGTTAG3′ 5′ACCAGCACACAGCATTACC3′ | 66 | *Aci*I |
| A3352G (polyA) | Gieselmann et al., Proc. Natl. Acad. Sci., 1989 | ARSA3114/ ARSA3417 | 5′CTGCTCAAGGCCCAGTTAG3′ 5′ACCAGCACACAGCATTACC3′ | 66 | *Mae*III |
| A2417G (N350S) | Gieselmann et al., Proc. Natl. Acad. Sci., 1989 | ARSA2298/ ARSA2434 | 5′CCTTGATGGCGAACTGAG3′ 5′AGCCATCCAAGGTGACAC3′ | 58 | Specific PCR |
| | | ARSA2298/ ARSA2434 | 5′CCTTGATGGCGAACTGAG3′ 5′AGCCATCCAAGGTGACAT3′ | 58 | Specific PCR |

non-Europeans [4], strongly suggests a single ancient origin posterior to the divergence of European and Asian populations. The identification of the singly mutated alleles on different haplotypes may suggest the existence of de novo mutation events. Allele 2417G/3352A appeared on six different haplotypes, although half of the alleles have the same haplotype (*Bgl*I (T) *Bsr*I (G) *Bam*HI (G) *Nla*III[2] (G) *Nla*III[1] (G) *Aci*I (A)), which was absent from the control alleles. Analysis of the heterozygote individual with alleles 2417G/3352G and 2417A/3352G revealed an haplotype (*Bgl*I (G, G) *Bsr*I (C, C) *Bam*HI (C, C) *Nla*III[2] (G, G) *Nla*III[1] (G, G) *Aci*I (G, G)) that is different from the one previously reported for another similar singly mutated allele [5]. The existing data does not allow us to exclude the possible existence of de novo mutations on different haplotype backgrounds nor the possible divergence of the polymorphic backgrounds of the mutated alleles.

The possible association between ARSA-PD and different neuro-psychiatric conditions has been investigated for many years, but with the exception of two alcoholism-related studies [6,7], in which a link is established between this condition and the ARSA-PD allele 2417G/3352A, all the other studies were done only by biochemical determination of ARSA activity "in vitro" and led to inconclusive results. In this study, the possible association between ARSA-PD and schizophrenia was investigated by comparing the frequency of the ARSA-PD associated alleles in the healthy Portuguese population and in a group of Portuguese patients with the diagnosis of schizophrenia. Among these patients we found frequencies of 3% for allele 2417G/3352G and 21.2% for allele 2417G/3352A. Although these two alleles were in Hardy–Weinberg equilibrium in both the control and the schizophrenic population samples, allele 2417G/3352A has a statistically significant higher frequency in the latter group ($\chi^2$ test, $p < 0.05$), which supports the idea of an increased risk for 2417G/3352A allele carriers to develop schizophrenia. This same allele has previously been reported as having a higher frequency among alcoholic patients [6,7].

Therefore, in addition to the diagnosis problems resulting from the low ARSA activity not associated with MLD clinical symptoms, there is also the possibility of ARSA-PD contributing to the susceptibility of development of neuro-psychiatric conditions. Moreover, it has been suggested that a reduced activity of ARSA, in the presence of detrimental stimuli such as lead [8] or alcohol [9], could lead to accumulation of sulfatide in cells of the nervous system resulting in impaired function. Thus, it seems that ARSA-PD may have important clinical implications, justifying the studies on this subject, which we are pursuing.

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## References

[1] K. von Figura, V. Gieselmann, J. Jaeken, Metachromatic leukodystrophy, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, 2001, pp. 3695–3724.

[2] V. Gieselmann, A. Polten, J. Kreysing, K. von Figura, Arylsulfatase A pseudodeficiency: loss of a polyadenylylation signal and a N-glycosylation site, Proc. Natl. Acad. Sci. USA 86 (23) (1989) 9436–9440.

[3] J. Zlotogora, Y. Furman-Shaharabani, S. Goldenfum, B. Winchester, K. von Figura, V. Gieselmann, Arylsulfatase A pseudodeficiency: a common polymorphism which is associated with a unique haplotype, Am. J. Med. Genet. 52 (2) (1994) 146–150.

[4] R. Ott, J.S. Waye, P.L. Chang, Evolutionary origins of two tightly linked mutations in arylsulphatase-A pseudodeficiency, Hum. Genet. 101 (2) (1997) 135–140.

[5] M.H. Ricketts, D. Goldman, J.C. Long, P. Manowitz, Arylsulphatase A pseudodeficiency-associated mutations: population studies and identification of a novel haplotype, Am. J. Med. Genet. 67 (4) (1996) 387–392.

[6] D.S. Park, R.D. Poretz, S. Stein, R. Nora, P. Manowitz, Association of alcoholism with the N-glycosylation polymorphism of pseudodeficient human arylsulfatase A, Alcohol. Clin. Exp. Res. 20 (2) (1996) 228–233.

[7] I.W. Chang, H. Kim, S.I. Lee, J.B. Hong, Y.D. Kim, H.M. Nan, W. Sribney, Y.S. Kim, P. Manowitz, Evidence for an N-glycosylation polymorphism of Arylsulfatase a predisposing to alcoholism in Koreans, Am. J. Med. Genet. 114 (2) (2002) 186–189.

[8] R.D. Poretz, A. Yang, W. Deng, P. Manowitz, The interaction of lead exposure and arylsulfatase A genotype affects sulfatide catabolism in human fibroblasts, Neurotoxicology 21 (3) (2000) 379–387.

[9] A.R. Hulyalkar, R. Nora, P. Manowitz, Arylsulfatase A variants in patients with alcoholism, Alcohol. Clin. Exp. Res. 8 (3) (1984) 337–341.

# CHAPTER 2. IMPACT OF ARSA MUTATIONS IN THE STRUCTURE/ FUNCTION OF THE ENZYME

## 2A. BIOCHEMICAL CHARACTERIZATION OF ARSA MUTATIONS

### 2A.1. BIOCHEMICAL CHARACTERIZATION OF ARSA MISSENSE MUTATIONS ASSOCIATED WITH LATE INFANTILE AND JUVENILE CLINICAL PHENOTYPES

### 2A.2. BIOCHEMICAL CHARACTERIZATION OF A NEW ARSA GENE MUTATION ASSOCIATED WITH AN ADULT ATYPICAL MLD CLINICAL PHENOTYPE

## 2B. IMPACT OF ARSA MISSENSE MUTATIONS IN THE ENZYME OLIGOMERIZATION CAPACITY

# 2A. BIOCHEMICAL CHARACTERIZATION OF ARSA MUTATIONS

# 2A.1. BIOCHEMICAL CHARACTERIZATION OF ARSA MISSENSE MUTATIONS ASSOCIATED WITH LATE INFANTILE AND JUVENILE CLINICAL PHENOTYPES

American Journal of Medical Genetics 116A:238–242 (2003)

# Biochemical Characterization of Two (C300F, P425T) Arylsulfatase A Missense Mutations

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**Metachromatic leukodystrophy (OMIM 250100) is a lysosomal storage disease caused by the deficiency of arylsulfatase A (ARSA, EC 3.1.6.8). This disease affects mainly the nervous system, because patients cannot degrade 3-*O*-sulfo-galactosylceramide (sulfatide), a major myelin lipid. Here we describe the characterization of the biochemical effects of two arylsulfatase A missense mutations, P425T and C300F. Transfection experiments demonstrate the expression of residual ARSA enzyme activity for P425T, but not for C300F substituted ARSA. Relative specific activity determination showed that the P425T substituted enzyme has retained about 12% of specific enzyme activity, whereas the C300F substituted enzyme is reduced to less than 1%. Pulse-chase experiments reveal that both mutant proteins are unstable, with a half life of less than 6 hr. Increased secretion upon addition of $NH_4Cl$ indicates that the mutant proteins can pass the Golgi apparatus and thus are not degraded in the endoplasmic reticulum (ER), but in the lysosomes. This is supported by experiments, which demonstrate the presence of mannose-6-phosphate residues on the oligosaccharide side chains of the mutant proteins. Addition of the cysteine protease inhibitor leupeptin increases the amount of ARSA activity in cells expressing the P425T substituted enzyme, whereas no increase in activity was seen with C300F substituted ARSA.** © 2002 Wiley-Liss, Inc.

**KEY WORDS: arylsulfatase A; metachromatic leukodystrophy; lysosomal storage disease**

## INTRODUCTION

Arylsulfatase A is the lysosomal enzyme that catalyzes the first step in the degradation of sulfatide (3-*O*-sulfogalactosylceramide), one of the major sphingolipids in myelin. Deficiency of this enzyme causes metachromatic leukodystrophy, a disease that is characterized by sulfatide accumulation in different tissues. Oligodendrocytes and Schwann cells are the most affected. Thus, patients develop a progressive demyelination and a wide variety of neurological symptoms. metachromatic leukodystrophy (MLD) is clinically heterogeneous, presenting with three different forms classified according to the age of onset: late infantile, juvenile, and adult forms [for review see Von Figura et al., 2001].

The ARSA locus is located on chromosome 22q13; the gene is divided into eight exons encompassing 3.2 Kb (GenBank X52150). It codes for an enzyme of 507 amino acids [Stein et al., 1989; Kreysing et al., 1990]. A large number of mutations has been reported in the ARSA gene. Most are private and only a small number of them were biochemically characterized [Von Figura et al., 2001].

A genotype–phenotype correlation exists in MLD: patients homozygous for alleles associated with no residual activity usually present the late infantile form of the disease, while patients with at least one allele or two alleles coding for an enzyme with some residual activity present less severe, juvenile or adult forms. This correlation was first suggested by Polten [Polten et al., 1991] and has been supported by biochemical data from several reports [Bohne et al., 1991; Fluharty et al., 1991; Leinekugel et al., 1992; Kappler et al., 1994; Hess et al., 1996; Coulter-Mackie et al., 1997; Hermann et al., 2000].

In this article, two defective ARSA proteins are biochemically characterized. Activity, stability, and sorting of these enzymes are analyzed and the obtained data are discussed according to the clinical phenotype of the patients.

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## MATERIALS AND METHODS

### Patients

The mutations characterized in this study were found in two Portuguese patients. One of these patients presented the late infantile clinical form of MLD and was homozygous for the alteration that changes cysteine 300 into phenylalanine. The other patient presented the juvenile form of the disease and was a compound heterozygote for mutation P426L [Polten et al., 1991] and a mutation that changes proline 425 into threonine. Both of these patients were previously reported [Marcão et al., 1999].

### Material

Taq polymerase, LIPOFECT AMINE Reagent, G418, and cell culture media were from Life Technologies (Eggenstein, Germany). Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). Restriction enzymes and DNA modifying enzymes were from New England Biolabs (Frankfurt am Main, Germany) or Life Technologies. Endoglycosidase H was from Roche. [$^{35}$S]-methionine (specific activity >600Ci/mmol) and [$^{32}$P]-orthophosphate (specific activity >3,000 Ci/mmol) were from Amersham Pharmacia (Freiburg, Germany). PANSORBIN was from Calbiochem-Novabiochem (Schwalbach, Germany). Other reagents were from Sigma or Merck.

### In Vitro Mutagenesis

Mutations C300F and P425T were introduced into the pAlter mutagenesis vector from Promega according to the protocol supplied by the manufacturer. The insert of the vector contained an SV40 early promoter, the ARSA cDNA and a polyadenylation sequence from the pBEH ARSA vector described previously [Artelt et al., 1988; Stein et al., 1989]. The oligonucleotides that were used to introduce mutations have the following sequences: C300F-5′TGCTCCGGACTCTTGCGGTTTGGAAAGGG-A3′ and P425T-5′ACTGCTCATGAGACCCCGCTCC-TCTATGAC3′. The introduction of these mutations was confirmed by DNA sequencing.

### Activity, Stability and Intracellular Localization of Mutant ARSAs

BHK-21 cells were transiently transfected with the mutagenesis vector using lipofectamine, as described [Hermann et al., 2000]. After 48 hr, the cells were harvested and ARSA activity was measured in cellular extracts using the artificial substrate *p*-nitrocatechol-sulphate. Total protein determination and Western blot analysis were also done.

The mutated ARSA cDNAs were subcloned into the expression vector pBEH [Artelt et al., 1988]. A neomycin cassette, containing a neomycin resistance gene with an SV40 promoter and a polyadenylation signal was inserted at the unique Aat II restriction site. To generate stably transfected cell lines, Ltk$^-$ cells were transfected, using lipofectamine, and selected for two weeks with 1mg/ml G418. After this time, metabolic labeling, immunoprecipitation, and digestion of ARSA with endoglycosidase H were done, as described before [Gieselmann et al., 1992], with minor modifications. An ARSA anti-serum, raised against recombinant ARSA in rabbits, was used.

## RESULTS

### ARSA Activity in Transiently Transfected BHK-21 Cells

The mutations causing the P425T and C300F amino acid substitutions have recently been described, but their deleterious effect on ARSA activity expression has not been confirmed. To verify that the mutations are pathologic, amino acid substitutions P425T and C300F were introduced into the normal ARSA cDNA by in vitro mutagenesis. The mutated cDNAs were transiently expressed in BHK-21 cells.

Five independent transient transfections were done for each mutation.

Figure 1 shows that enzyme activity expression is severely reduced for mutant P425T and completely absent for mutant C300F. To investigate ARSA relative specific activities (Table I), we determined the enzyme activity and the amount of ARSA cross-reacting material via Western blot analysis in the same cell lysates. The results reveal that the P425T substituted protein has considerable residual specific activity of about 12% of normal, whereas the specific activity of the C300F substituted ARSA is less than 1%.

It has previously been demonstrated that some mutant ARSA proteins can be stabilized in cultured cells by the addition of the protease inhibitor leupeptin [Von Figura et al., 1983]. Upon addition of leupeptin the

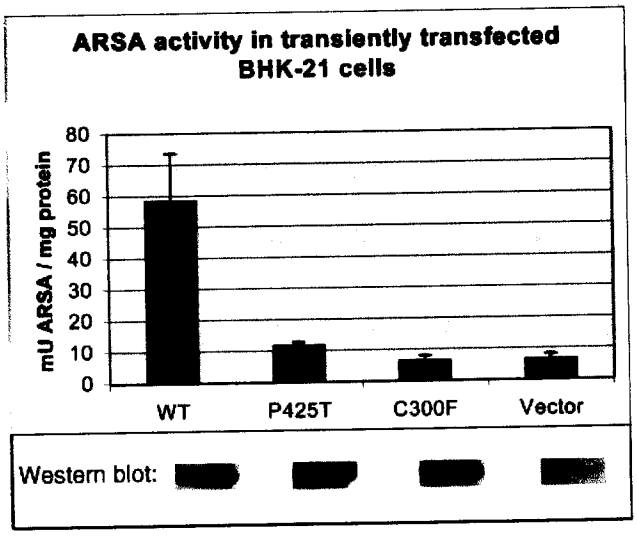


Fig. 1. ARSA activity in transiently transfected BHK-21 cells. ARSA activity was calculated as the average of five independent transfection experiments and is expressed as mU ARSA/mg total protein. Bars indicate standard deviation. To determine endogenous activity of BHK-21 cells, the same vector, but with an insert encoding a nonrelated protein, was also transfected and ARSA activity was measured. The bottom shows the result of a Western blot analysis of cell homogenates of transfected cells confirming the presence of ARSA cross-reacting material.

TABLE I. ARSA Relative Specific Activity*

| | WT | P425T | C300F | BHK-21 |
|---|---|---|---|---|
| ARSA activity–ARSA endogenous activity (μU) | 301.5 | 39 | 3 | 0 |
| Western blot arbitrary units | 2,427 | 2,654 | 4,216 | 0 |
| Relative specific activity | 100% | 12% | 0.6% | — |

*To estimate the relative specific activities of the mutant enzymes, transiently transfected BHK-21 cells were analyzed for ARSA activity and ARSA cross-reacting material, determined by Western blot analysis. Western blot signals were quantified by laser scan densitometry of the X-ray films and are presented in arbitrary units. Relative specific activity was calculated as the quotient between ARSA activity and the Western blot signal.

ARSA activity in the cells increases and the catabolism of sulfatide is normalized. To investigate whether the P425T and C300F substituted enzymes can also be stabilized by cysteine protease inhibitors, leupeptin was added to transiently transfected cells and enzyme activity was determined. Table II shows that addition of leupeptin leads to a considerable increase of enzyme activity in the cells expressing the P425T substituted enzyme. However, the protease inhibitor has no influence on ARSA activity in cells expressing the C300F substituted ARSA.

### Stability and Intracellular Localization of the Mutant ARSA Polypeptides

To investigate the effects of the amino acid substitutions on the stability of the mutant proteins, pulse chase experiments were performed.

Stably transfected Ltk$^-$ cells expressing wild-type ARSA, C300F, and P425T substituted ARSA were pulse-labeled for 3 hr with [$^{35}$S]-methionine and chased up to 72 hr.

Figure 2 shows that the wild-type enzyme is stable during the entire chase period, whereas both mutant enzymes are rapidly degraded. More than half of the mutated proteins are already degraded after 6-hr chase, an almost complete degradation occurring after 24 hr.

Premature degradation of mutant ARSA can either occur in the endoplasmic reticulum (ER) or in the lysosomes. To investigate whether the mutated proteins can leave the ER, pulse-chase experiments in the presence of $NH_4Cl$ were done. $NH_4Cl$ interferes with the cellular sorting of newly synthesized lysosomal enzymes in a post Golgi compartment, targeting them improperly to the secretory pathway. If upon addition of $NH_4Cl$ mutant proteins can be found in the media of cultured cells, this indicates that these enzymes must have passed the Golgi apparatus and thus are able to leave the ER [Chang et al., 1988]. Cells were pulse labeled with [$^{35}$S]-methionine and chased in the absence or presence of $NH_4Cl$. ARSA was immunoprecipitated from cells and media. The amount of C300F and P425T substituted ARSA secreted into the medium increases in the presence of ammonium chloride (Fig. 3) which indicates that the mutant proteins are not arrested in the ER.

The synthesis of the mannose 6-phosphate residues on the oligosaccharide side chains of lysosomal enzymes occurs in the Golgi apparatus. Thus, demonstration of the presence of phosphate residues on N-linked oligosaccharide side chains is another proof for Golgi passage of a lysosomal enzyme. Ltk$^-$ cells expressing the mutated proteins and the wild-type ARSA were pulse-labeled for 5 hr with [$^{32}$P]-orthophosphate. After immunoprecipitation the proteins were digested with endoglycosaminidase H, which removes high mannose-type oligosaccharide side chains.

Figure 4 shows that both mutant enzymes are labeled by [$^{32}$P]. The radioactive label can be removed by EndoH digestion, indicating its presence on high mannose type N-linked oligosaccharide side chains.

## DISCUSSION

We biochemically characterized two missense mutations in the ARSA gene. The mutations have been

TABLE II. Effect of Leupeptin in the ARSA Activity of Transiently Transfected BHK-21 Cells

| | ARSA activity (mU/mg protein | |
|---|---|---|
| | Leupeptin (100 μM) | |
| Expressed protein | – | + |
| LAP | 11.40 | 8.90 |
| ARSA-C300F | 7.77 | 10.64 |
| ARSA-P425T | 11.89 | 45.85 |
| ARSA-WT | 47.55 | 46.35 |

LAP, lysosomal acid phosphatase. BHK-21 cells were transiently transfected to express the wild-type or substituted ARSA indicated in the table. Control cells were transfected with a plasmid coding for lysosomal acid phosphatase (LAP) Leupeptin (100 μM) was added after the transfection. The cells were harvested and ARSA activity and protein were measured in cell homogenates, 48 hr, after transfection.

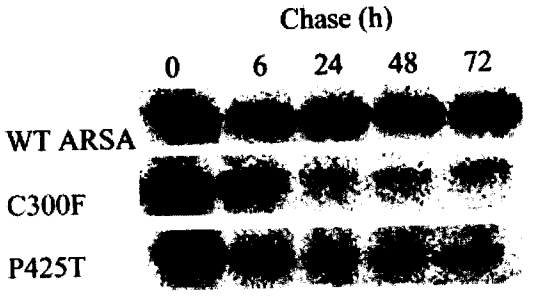


Fig. 2. Stability of wild-type and mutated ARSA polypeptides. Stable transfected Ltk$^-$ cells expressing wild-type or C300F and P425T substituted ARSA were metabolically labeled for 3 hr with [$^{35}$S]-methionine and chased for the times indicated. ARSA was immunoprecipitated from the cell lysates and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography.

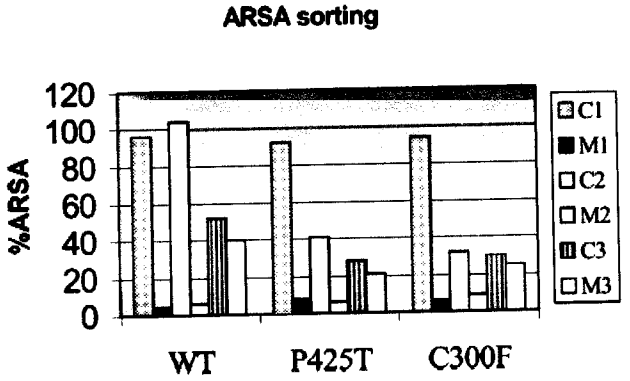


Fig. 3. Effect of $NH_4Cl$ on sorting of wild-type and mutant ARSA polypeptides. Ltk$^-$ cells stably expressing the wild-type or the mutant proteins were pulse-labeled for 2 hr with [$^{35}$S]-methionine and chased for 12 hr in the presence or in the absence of 10 mM $NH_4Cl$. After immunoprecipitation of ARSA from the medium and the cells, ARSA was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography. The bars indicate the percentage of ARSA cross-reacting material found in the cells (C) or in the media (M) and show the average of two independent experiments. 1, no chase; 2, 12-hr chase; 3–12-hr chase in the presence of $NH_4Cl$. For each protein, 100% ARSA = C1 + M1.

reported previously but their biochemical consequences have not been examined so far. Here we show that about 10% residual enzyme activity can be expressed from the P425T allele but not from the C300F allele. This is in accordance with the finding that the P425T ARSA has considerable residual specific enzyme activity, whereas that of the C300F is at the limit of detection (Fig. 1, Table I).

Biosynthesis studies show that both mutant enzymes are more rapidly degraded than the wild-type ARSA. The latter is stable over a chase period of 72 hr, whereas the mutant enzymes are partially and completely degraded after 6 hr and 24 hr of chase, respectively (Fig. 2).

Decreased stability of mutant ARSA frequently contributes to the enzyme deficiency in MLD [Kafert et al., 1995; Hess et al., 1996]. Previous studies have shown that the degradation of the mutant enzymes occurs either in the ER [Kafert et al., 1995; Hess et al., 1996] or in the lysosomes [Von Figura et al., 1983; Kreysing et al., 1993]. This means that the structural alterations resulting from mutations in this protein, lead either to endoplasmic reticulum retention and degradation

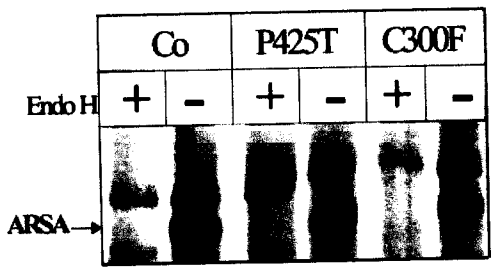


Fig. 4. Phosphorylation of mutated ARSA polypeptides. Ltk$^-$ cells, expressing wild-type or substituted ARSA polypeptides, were metabolically labeled for 5 hr with [$^{32}$P]-orthophosphate. ARSA was immunoprecipitated from the cell lysates and digested with endoglycosidase H before sulfate–polyacrylamide gel electrophoresis analysis.

[Kafert et al., 1995; Hess et al., 1996] or cause an increased sensitivity for lysosomal proteases [Von Figura et al., 1983; Kreysing et al., 1993]. Here we demonstrate an increased secretion upon addition of $NH_4Cl$ of both mutant enzymes (Fig. 3). Since this drug interferes with lysosomal enzyme sorting in a post Golgi compartment, this suggests that these enzymes are not arrested in the ER. This is supported by the presence of mannose 6-phosphate residues on the oligosaccharide side chains of the mutant enzymes (Fig. 4), because these residues are generated in a post ER compartment.

The biochemical properties of the P425T mutation resemble those of a previously described amino acid substitution, which occurs in the neighboring amino acid, P426L. The latter is one of the most frequent mutations found among whites [Polten et al., 1991]. The P426L substituted enzyme is correctly synthesized and sorted, but is rapidly degraded in the lysosomes. This degradation could also be partially blocked by leupeptin [Von Figura et al., 1983]. At neutral pH ARSA is a dimer that octamerizes in a pH dependent manner in the acidic environment of the lysosome. Octamerization of the P426L substituted enzyme is impaired and this allows cathepsin L to cleave the mutant enzyme [von Bülow, 2002]. It seems likely that the P425T substitution described here, may also interfere with octamerization explaining its cysteine protease sensitivity. Thus, this mutation has biochemical properties, which are similar to those of the P425T mutation described here. Prolines 425 and 426 are part of a PPLL motive that is strongly conserved among different sulfatases [Von Figura et al., 2001]. Thus, it seems that independent of the mutations, alterations of this motif have similar consequences, namely, increased intralysosomal susceptibility for cysteine proteinases.

The biochemical data presented here fit well into the established genotype–phenotype correlation of MLD. The juvenile patient carrying the frequent P426L mutation in one allele was found to be a compound heterozygote for P425T mutation in ARSA's exon 8. Both mutations still allow for the expression of low residual enzyme activities and thus, explain the late-onset phenotype of the patient.

The C300F allele was found in homozygosity in a patient with the severe late infantile form of the disease. We have shown that no enzyme activity can be expressed from the allele carrying this mutation. This enzyme is also sorted correctly to the lysosomes and thus resembles the P425T ARSA. Since it also displays a comparable stability in pulse-chase experiments, the main reason for the difference in enzyme activity must be the reduced specific activity of the enzyme. Therefore, the addition of leupeptin cannot increase the ARSA activity in cells expressing this mutant, because in contrast to the P425T substituted enzyme, C300F is enzymatically inactive (Fig. 1, Table I).

## ACKNOWLEDGMENTS

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## REFERENCES


Artelt P, Morelle C, Ausmeier M, Fitzek M, Hauser H. 1988. Vectors for efficient expression in mammalian fibroblastoid, myeloid and lymphoid cells via transfection or infection. Gene 68:213–219.

Bohne W, von Figura K, Gieselmann V. 1991. An 11-bp deletion in the arylsulfatase A gene of a patient with late infantile metachromatic leukodystrophy. Hum Genet 87:155–158.

Chang PL, Ameen M, Yu CZ, Kelly BM. 1988. Effect of ammonium chloride on subcellular distribution of lysosomal enzymes in human fibroblasts. Exp Cell Res 176:258–267.

Coulter-Mackie MB, Gagnier L, Beis MJ, Applegarth DA, Cole DE, Gordon K, Ludman MD. 1997. Metachromatic leukodystrophy in three families from Nova Scotia, Canada: a recurring mutation in the arylsulfatase A gene. J Med Genet 34:493–498.

Fluharty AL, Fluharty CB, Bohne W, von Figura K, Gieselmann V. 1991. Two new arylsulfatase A (ARSA) mutations in a juvenile metachromatic leukodystrophy (MLD) patient. Am J Hum Genet 49:1340–1350.

Gieselmann V, Schmidt B, Von Figura K. 1992. In vitro mutagenesis of potential N-glycosylation sites of arylsulfatase A. J Biol Chem 267: 13262–13266.

Hermann S, Schestag F, Polten A, Kafert S, Penzien J, Zlotogora J, Baumann N, Gieselmann V. 2000. Characterization of four Arylsulfatase A missense mutations G86D, Y201C, D255H, and E212D causing metachromatic leukodystrophy. Am J Med Genet 91:68–73.

Hess B, Kafert S, Heinisch U, Wenger DA, Zlotogora J, Gieselmann V. 1996. Characterization of two arylsulfatase A missense mutations Asp335>Val and Thr274>Met causing late infantile metachromatic leukodystrophy. Hum Mutat 7:311–317.

Kafert S, Heinisch U, Zlotogora J, Gieselmann V. 1995. A missense mutation P136L in the aylsulfatase A gene causes instability and loss of activity of the mutant enzyme. Hum Genet 95:201–204.

Kappler J, Sommerlade H, von Figura K, Gieselmann V. 1994. Complex arylsulfatase A alleles causing metachromatic leukodystrophy. Hum Mutat 4:119–127.

Kreysing J, von Figura K, Gieselmann V. 1990. Structure of the arylsulfatase A gene. Eur J Biochem 191:627–631.

Kreysing J, Bohne W, Bosenberg C, Marchesini S, Turpin JC, Baumann N, von Figura K, Gieselmann V. 1993. High residual arylsulfatase A (ARSA) activity in a patient with late-infantile metachromatic leukodystrophy. Am J Hum Genet 53:339–346.

Leinekugel P, Michel S, Conzelmann E, Sandhoff K. 1992. Quantitative correlation between the residual activity of β-hexosaminidase A and arylsulfatase A and the severity of the resulting lysosomal storage diseases. Hum Genet 88:513–523.

Marcão A, Amaral O, Pinto E, Pinto R, Sá Miranda MC. 1999. Metachromatic leucodystrophy in Portugal—finding of four new molecular lesions: C300F, P425T, g.1190-1191insC, and g.2408delC. Hum Mut 13:337–338.

Polten A, Fluharty AL, Fluharty CB, Kappler J, von Figura K, Gieselmann V. 1991. Molecular basis of different forms of metachromatic leukodystrophy. N Engl J Med 324:18–22.

Stein C, Gieselmann V, Kreysing J, Schmidt B, Pohlmann R, Waheed A, Meyer H, O'Brien J, Von Figura K. 1989. Cloning and expression of human arylsulfatase A. J Biol Chem 264:1252–1259.

von Bülow R, Schmidt B, Dierks T, Schwabauer N, Schilling K, Weber E, Uson I, von Figura K. 2002. Defective oligomerization of arylsulfatase A as a cause of its instability in lysosomes and metachromatic leukodystrophy. J Biol Chem 277:9455–9461.

Von Figura K, Steckel F, Hasilik A. 1983. Juvenile and adult metachromatic leukodystrophy: partial restoration of arylsulfatase A (cerebroside sulfatase) activity by inhibitors of thiol proteinases. Proc Natl Acad Sci USA 80:6066–6070.

Von Figura K, Gieselmann V, Jaeken J. 2001. Metachromatic leukodystrophy. In: Scriver CR, et al., editor. The metabolic and molecular basis of inherited disease. 8th edition. New York: McGraw-Hill. p 3695–3724.

# 2A.2. BIOCHEMICAL CHARACTERIZATION OF A NEW ARSA GENE MUTATION ASSOCIATED WITH AN ADULT ATYPICAL MLD CLINICAL PHENOTYPE

**ADULT ONSET MLD WITHOUT PERIPHERAL NERVOUS SYSTEM INVOLVEMENT: A CASE WITH A NEW MUTATION IN THE ARSA GENE**

Marcão A, Wiest R, Schindler K, Wiesmann U, Schroth G, Sá Miranda MC, Sturzenegger M, Gieselmann V

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**Abstract**

We report the case of a 37-year old woman of tamile origin who presented with rapidly progressive dementia and behavioural abnormalities due to adult onset Metachromatic Leucodystrophy (MLD), a disease that usually affects the central and peripheral nervous system. As expected, MRI of the patient showed severe subcortical brain atrophy and extended T2 hyperintense signal alterations of white matter. Surprisingly, the cerebellar white matter was not affected and the patient did not show any clinical, neurophysiological or pathological signs of peripheral nervous system dysfunction. In accordance with these findings the patient did not present with ataxia or symptoms of peripheral neuropathy, which are frequent in MLD. Genetic analysis revealed homozygosity for a novel mutation in exon 3 of the arylsulfatase A gene (F219V). This substitution leads to a misfolded, unstable enzyme with a specific activity of less than 1% of normal. This results in a severe enzyme deficiency, which is unusual in adult patients.

**Introduction**

Metachromatic Leukodystrophy (MLD; OMIM 250100) is an inherited lysosomal storage disorder caused by the deficiency of the Arylsulfatase A (ARSA; E.C.

3.1.6.8). ARSA catalyses the first step in the intralysosomal degradation of 3-O-sulfo-galactosylceramide (sulfatide), namely the desulfation of the galactose moiety of this sphingolipid. Sulfatide is found in various tissues like the distal tubules of the kidney, bile duct epithelia and in particularly high concentrations in myelin of the nervous system. ARSA deficiency causes intralysosomal accumulation of sulfatide, which affects the oligodendrocytes and Schwann cells leading to progressive demyelination in the central and peripheral nervous system, respectively (for review see 1).

Three forms of MLD, according to the age of onset, can be distinguished: a late infantile form with onset at the age of 1-2 years and early death of the individual; a juvenile form starting at the age of 3 to 16 years; and an adult form (onset >16 years) with a more protracted course[1]. A genotype–phenotype correlation has been described for MLD: patients who are homozygous for alleles which do not allow for the expression of any residual enzyme activity suffer from the most severe, late infantile form of the disease, whereas homozygosity for alleles allowing for the expression of residual enzyme activity in many cases causes the mild adult forms of the disease. Patients heterozygous for these two kinds of alleles frequently suffer from the intermediate juvenile MLD[2, 3].

Up to now, more than 90 different mutations causing the disease have been described in the ARSA gene (1 and *www.hgmd.org*). Only few of these alleles are frequent among patients, whereas most mutations are unique to individual families[1].

Here we present an unusual case of adult MLD caused by a novel mutation in the exon 3 of the arylsulfatase A gene (F219V).

## Patient and Methods

### Case report

A 37-year-old woman, whose parents were related and of tamile origin, presented with inadequate and bizarre behaviour after birth of her third child in 1997. She had been working as a housewife since her emigration to Switzerland in 1989 and was described as well organised and intelligent. She had been trained as a primary school teacher in Sri Lanka and worked without problems as a teacher until 1989. In 1997 she showed progressive apathy, loss of interest in daily living routines and care of her three children. Memory functions deteriorated dramatically and she developed bladder incontinence. In May 1997 she was referred to the hospital because of a fall that originated a head injury. At that time, CT scans showed moderate ventricular enlargement, extended and marked white matter lesions. Further evaluation revealed severely impaired cognitive and mental functions, but there were no signs of peripheral nervous system involvement, a sural nerve biopsie as well as the electrophysiological studies revealed normal. MRI studies showed severe subcortical brain atrophy and extended T2 hyperintense signal alterations, nevertheless the cerebellar white matter was not affected.

### Materials

Taq polymerase, LIPOFECTAMINE™ Reagent, G418 and cell culture media were from Life Technologies (Eggenstein, Germany). Oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). Restriction enzymes and DNA modifying enzymes were from New England Biolabs (Frankfurt am Main, Germany) or Life Technologies. [$^{35}$S]-methionine (specific activity > 39 TBq/mmol) was from Amersham

Pharmacia (Freiburg, Germany). PANSORBIN® was from Calbiochem-Novabiochem (Schwalbach, Germany). Other reagents were from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany).

**DNA sequence analysis**

The ARSA gene of the patient was amplified in two overlapping fragments and sequenced completely. Amplification, sequencing strategy and sequences of primers used for amplification and sequencing have been described in detail[4].

**"In vitro" mutagenesis**

Mutation F219V was introduced into the ARSA cDNA[5] by site directed mutagenesis. For that purpose a fragment from the vector pBEH-ARSA containing the SV40 early promoter, the ARSA cDNA and a polyadenylation sequence[5, 6] was cloned in the pALTER mutagenesis vector from Promega. "In vitro" mutagenesis was performed according to the protocol supplied by the manufacturer. The oligonucleotide that was used for the introduction of the F219V mutation had the sequence: 5'-GATCGCCCCGTCTTCCTGTAC-3'. The introduction of this mutation was confirmed by DNA sequencing.

**Transfections, metabolic labelling and immunoprecipitation of mutant ARSAs**

BHK cells were transiently transfected with the mutagenesis vector using lipofectamine, as described[7]. After 48h, the cells were harvested and ARSA activity was measured in cellular extracts using the artificial substrate p-nitrocatecholsulphate as described[8]. Protein was determined with the Biorad DC protein assay kit according to

the protocol supplied by the manufacturer. Western blot analysis was performed with an ARSA antiserum raised against recombinant ARSA in rabbits.

For the generation of stably transfected Ltk$^-$ cell lines, the mutated ARSA cDNA was subcloned into the expression vector pBEH[6]. A neomycine cassette, containing a neomycin resistance gene with an SV40 promoter and a polyadenylation signal, was inserted at the unique Aat II restriction site of the pBEH plasmid. The Ltk$^-$ cells were transfected using lipofectamine[7], and selected for two weeks with 1mg/ml G418. Metabolic labelling and immunoprecipitation of ARSA were done, as described previously in detail[9].

The generation and characterisation of monoclonal anti-ARSA antibodies has been described in reference 10.

## Results

### Consequences of the F219V substitution

The entire ARSA gene of the patient was amplified and sequenced completely. Only one sequence alteration was found in exon 3. A T > G transition at cDNA position 655 changes codon 219 from TTC coding for phenyalanine to GTC coding for valine. The patient is homozygous for this allele and no other mutation was found in the ARSA gene.

To prove that this missense mutation affects ARSA activity, expression vectors coding for the wild type, a P425T and a F219V substituted ARSA were transiently transfected into BHK cells. The P425T substitution has recently been found in a juvenile patient with MLD and has been shown to be associated with residual enzyme activity[11,12]. Forty-eight hours after transfection, ARSA activity was measured in the cell

lysates. Almost no activity was expressed from the plasmid containing the F219V substituted cDNA, whereas low levels of residual activity were found in lysates of cells expressing the P425T substituted enzyme (see Figure 1, top). Western blot analysis revealed the presence of ARSA cross reacting material in all transfected cells, which allows the conclusion that the F219V mutation severely reduces the specific enzymatic activity of ARSA (Figure 1, bottom, Table 1).

**Table 1 - ARSA relative specific activity**

| | WT | P425T | F219V | BHK |
|---|---|---|---|---|
| ARSAactivity-ARSAendogenous activity (μU) | 301,5 | 39 | 4,5 | 0 |
| Western-blot arbitrary units | 2427 | 2654 | 3707 | 0 |
| Relative specific activity | 100% | 12% | 1% | - |

To estimate the relative specific activities of the mutant enzymes, transiently transfected BHK cells were analysed for ARSA activity and ARSA cross-reacting material, determined by Western blot analysis. Western blot signals were quantified by laser scan densitometry of the X-ray films and are presented in arbitrary units. Relative specific activity was calculated as the quotient between ARSA activity and the Western blot signal. Results of P425T were already published in ref. 12. This mutation was used here as a control to allow direct comparison to the F219V mutation.

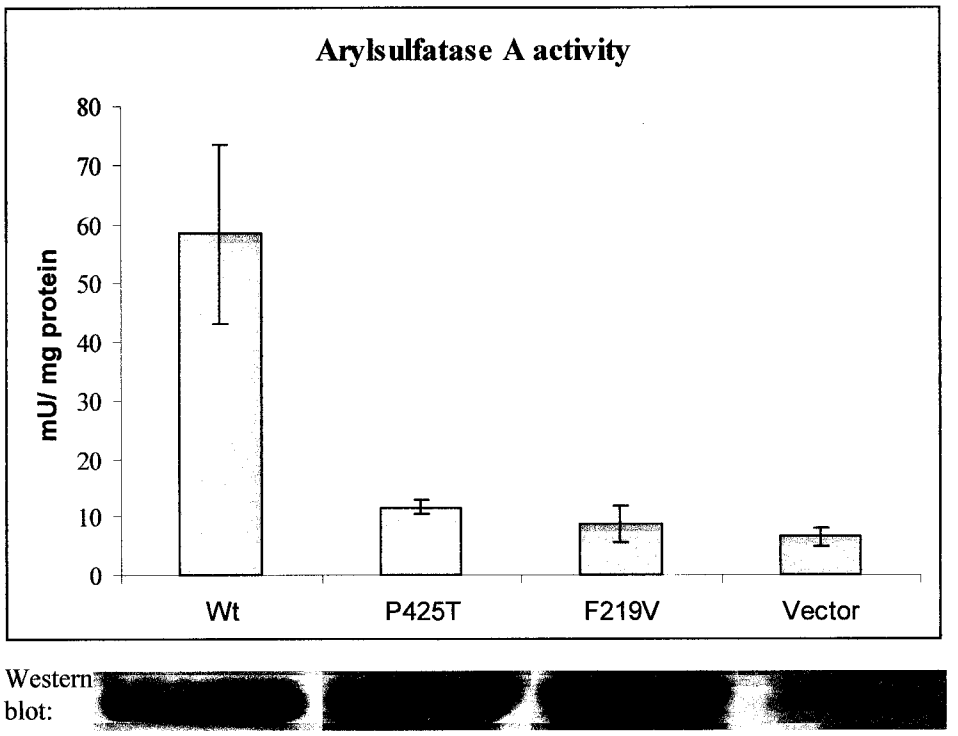


**Figure 1 - ARSA activity in transiently transfected BHK cells.** Wild-type (Wt), F219V and P425T substituted ARSA cDNAs were transiently expressed in BHK cells. Forty-eight hours after transfection ARSA activity was measured in the cell homogenates. ARSA activity was calculated as the average of five independent transfection experiments and is expressed as mU ARSA/ mg total protein. Bars show the standard deviation. To determine endogenous background sulfatase activity of BHK cells, the same vector, but with an insert encoding a non-related protein, was also transfected and ARSA activity measured. Results for the P425T mutation were published earlier[12], they were used here for direct comparison to the F219V mutation. The bottom of the figure shows the result of a Western blot analysis confirming the presence of ARSA cross-reacting material.

To investigate whether the F219V substitution also affects the stability of ARSA, pulse chase experiments with stably transfected cells were performed (see Figure 2). Whereas wild type ARSA was stable during the chase period of 72h, the

majority of the F219V substituted ARSA was degraded already after 6 h. Thus, this enzyme is highly unstable.

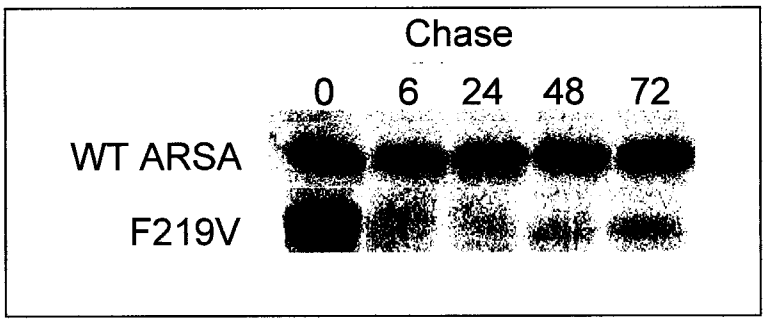


**Figure 2 - Stability of F219V-ARSA.** Stably transfected Ltk$^-$ cells expressing wild type or mutated ARSA were metabolically labelled for 3h with [$^{35}$S]-methionine and chased for the times indicated in hours. ARSA was immunoprecipitated from the cell lysates, subjected to SDS-PAGE and visualized by a phosphoimager.

To get a measure of the structural integrity of the F219V substituted enzyme, we immunoprecipitated wild type, F219V and P425T substituted ARSA from transiently transfected, metabolically labelled BHK cells with several monoclonal antibodies. These monoclonal antibodies have been shown to precipitate only the native non denatured enzyme[10]. Whereas the wild type ARSA and the P425T substituted enzyme can be precipitated with all monoclonal antibodies used, the F219V substituted ARSA could not be precipitated by any of these antibodies (see Figure 3).

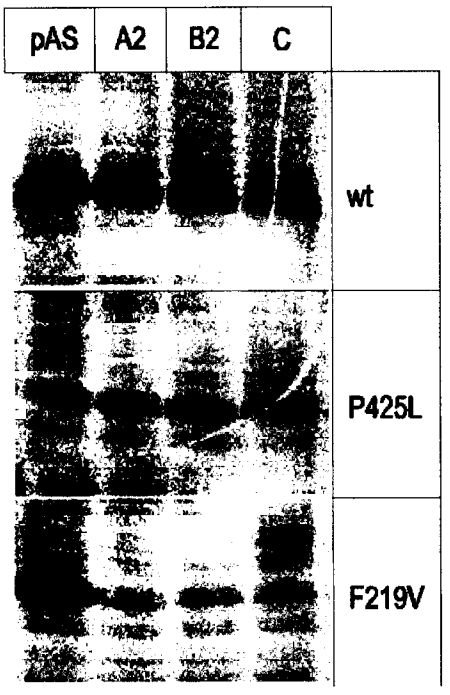


**Figure 3 - Reactivity of ARSA with monoclonal antibodies.** Wild type, F219V and P425T substituted ARSA cDNAs were transiently transfected in BHK cells. Forty-eight hours after transfection, cells were labelled for 3h with [$^{35}$S]-methionine. Cells were harvested and the cell homogenates were divided into four aliquots. From these aliquots ARSA was immunoprecipitated either by a polyclonal antiserum (pAS) or by the three structure sensitive monoclonal antibodies (A2, B2 and C), which recognize three different epitopes[10].

Degradation of mutant ARSA may occur in the ER and/or in the lysosomes. To investigate whether the F219V-ARSA can leave the ER, pulse-chase experiments were performed in the presence of $NH_4Cl$. $NH_4Cl$ interferes with the intracellular sorting of newly synthesised lysosomal enzymes in a post-Golgi compartment, targeting them improperly to the secretory pathway. If upon addition of $NH_4Cl$, mutant proteins can be detected in the media of cultured cells, this indicates that these enzymes must have passed the Golgi apparatus and thus, are able to leave the ER[13]. Cells were pulse labelled with [$^{35}$S]-methionine and chased in the absence or presence of $NH_4Cl$. ARSA was immunoprecipitated from cells and media (Figure 4). Without $NH_4Cl$ only little

cross-reacting material is found in the medium for cells expressing the wild type or the F219V-ARSA. Upon addition of $NH_4Cl$ about 50% of the wild type ARSA is found in the medium. In the presence of $NH_4Cl$ about 20% of the mutant F219V enzyme is secreted indicating that at least some of the enzyme can pass through the secretory pathway.

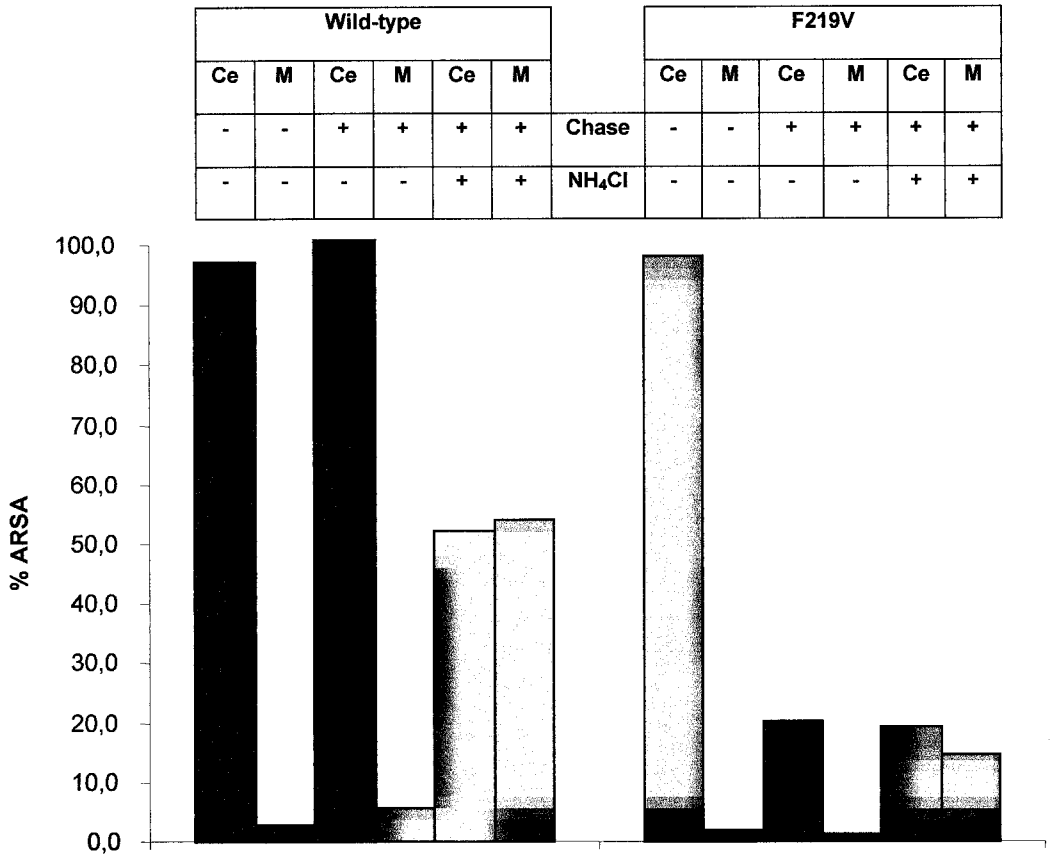


**Figure 4- Sorting of the mutant ARSA polypeptides.** Ltk$^-$ cells stably expressing the wild type or the F219V-ARSA were pulse-labelled for 2h with [$^{35}$S]-methionine and chased for 12h in the presence or in the absence of 10mM $NH_4Cl$. After immunoprecipitation, from the medium or from the cells, ARSA was subjected to SDS-PAGE and radioactive polypeptides were visualized and quantified by a phosphoimager. The presented results are the average of two independent experiments. The amount of ARSA cross reacting material which was found in the cells plus media after the 2h pulse period were taken as 100%.

**Discussion**

Metachromatic Leukodystrophy in adults usually starts between 16 and 30 years, but onset has been described up to an age of 60 years[14]. Initially, behavioural abnormalities and emotional lability, together with psychiatric symptoms such as delusions or hallucinations, are common. After months to years, spastic paresis of the extremities, exaggerated tendon reflexes and pyramidal signs may occur. Atactic symptoms, extrapyramidal signs, progressive dementia and clinical signs of peripheral neuropathy such as flaccid paresis usually occur[1]. Basically, two groups of adult patients can be distinguished: patients in whom initially psychiatric symptoms and impairment of intellectual functions dominate over neurologic symptoms and, in contrast, patients who present early with neurologic deficits. Patients with the former phenotype frequently are carriers of the I179S allele, suggesting a genotype-phenotype correlation in adult patients with respect to the initial symptoms that they develop[15, 16, 17]. Adult patients also show variability with respect to the extent of affection of the peripheral and central nervous system. Patients have been described, who presented with isolated peripheral neuropathy and lacked central nervous system dysfunction[18, 19, 20, 21]. In contrast, other patients in whom psychiatric and intellectual deficits dominate frequently miss clinical signs of peripheral neuropathy[22, 23]. Nevertheless, only few cases have been reported of patients with MLD and normal electrophysiological and histological results[24, 25]. The patient described here presented exclusively severe cognitive and intellectual impairment, 18 months after the onset of the disease, at the age of 37. She showed no signs of peripheral nervous system involvement. The sural nerve biopsies of the patient revealed no abnormalities and electrophysiological studies were within normal ranges on both upper and lower extremities. In addition, she was not atactic,

which is in accordance with the lack of lesions in the white matter of the cerebellum. Different kinetics of lysosomal storage of sulfatides in oligodendrocytes and Schwann cells, reduced segmental demyelination in the peripheral nerve and better tolerance of peripheral nerve tissues for sulfatide accumulation during adulthood or other genetic or epigenetic factors may be the reason for preserved function and normal morphology in rare instances.

This patient is homozygous for a new mutation causing the substitution of phenylalanine 219 by valine. The clinical heterogeneity of MLD can partially be ascribed to the amount of residual enzyme activity that is associated with the genotype of the patient[2,3]. In most cases, adult patients are homozygous for alleles displaying residual enzyme activity. Here we have compared the activity that can be expressed from a cDNA, which is F219V substituted, and another cDNA that is P425T substituted. The latter mutation was found in a patient with the juvenile form of the disease and has been shown to be associated with residual enzyme activity[11, 12]. Surprisingly, the F219V substitution causes reduction of enzyme activity below the levels of the P425T mutation and therefore to an extent unexpected for an adult MLD patient. A reduced specific activity and reduced stability of the F219V substituted enzyme account for this reduction. The influence of $NH_4Cl$ on the sorting of the mutant enzyme reveals that, at least a fraction of the mutant enzyme is able to leave the ER and pass the Golgi apparatus. Upon addition of $NH_4Cl$ to cells expressing the wild type enzyme, about 50% of ARSA cross-reacting material is found in the medium, whereas 50% remains inside the cell. In total, there is no loss of wild type ARSA polypeptides during the chase period. In contrast, after $NH_4Cl$ addition to cells expressing the F219V substituted enzyme, only about 40% of ARSA can be recovered (20% in the secretions, 20%

remaining intracellularly, see Figure 4). If the degradation of this mutant enzyme was exclusively lysosomal, one would have expected amounts in the medium that were comparable to the wild type enzyme. The low amount of enzyme found in the medium may indicate that the enzyme is partially retained and degraded in the ER.

The reasons for the discrepancy between the low enzyme activity and the mild phenotype of the patient remain unclear. Similar phenomena have been described in patients with early onset displaying exceptionally high residual enzyme activities[26]. Since the assays performed in vitro usually determine the enzyme activity with an artificial water-soluble substrate, it is likely that in some cases they do not reflect the in vivo situation in which an insoluble lipid substrate is degraded. On the other hand, it is known that genetic factors not linked to the ARSA locus can influence the phenotype of patients[27] and this may also be the case in the patient described here. Except for the low activity, other parameters also suggest that the F219V substitution affects the enzyme more severely than other adult type mutations. Thus, for the most frequent adult type P426L mutation it has been shown that the enzyme leaves the ER, is properly sorted in the Golgi apparatus and degraded in the lysosome[28]. The reason for the instability is probably the lack of octamerization, which usually occurs in the low pH environment of the lysosome[29]. The F219V enzyme, however, seems to be at least partially retained in the ER. We have also used various monoclonal antibodies, which epitopes depend on the correct three-dimensional folding of the enzyme. Whereas the wild type enzyme and the P425T substituted enzyme retain the epitopes, the F219V enzyme is not recognized by any of these antibodies. This indicates that this mutation causes a severe structural alteration of the enzyme. This result is in accordance with the low specific activity of the enzyme.

In summary, we describe an adult onset case of MLD, caused by a newly identified mutation in the arylsulfatase A gene, F291V. This patient has no clinical, electrophysiological or morphological signs of peripheral nerve affection and is homozygous for an allele that expresses only low amounts of residual enzyme activity.

**Acknowledgements**

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**References**

1. von Figura K, Gieselmann V, Jaeken J. Metachromatic leukodystrophy. In: Scriver CR, et al, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill, 2001:3695-3724
2. Polten A, Fluharty AL, Fluharty CB, et al. Molecular basis of different forms of metachromatic leukodystrophy. New Eng J Med 1991;324:18-22
3. Leinekugel P, Michel S, Conzelmann E, Sandhoff K. Quantitative correlation between the residual activity of β-hexosaminidase A and arylsulfatase A and the severity of the resulting lysosomal storage disease. Hum Genet 1992;88:513-520
4. Heinisch U, Zlotogora J, Kafert S, Gieselmann V. Multiple mutations are responsible for the high frequency of metachromatic leukodystrophy in a small geographic area. Am J Hum Genet 1995;56:51-57
5. Stein C, Gieselmann V, Kreysing J, et al. Cloning and expression of human arylsulfatase A. J Biol Chem 1989;264:1252-1259
6. Artelt P, Morelle C, Ansmeier M, et al. Vectors for efficient expression in mammalian fibroblastoids, myeloid and lymphoid cells via transfection or infection. Gene 1988;68:213-219
7. Hermann S, Schestag F, Polten A, et al. Characterization of four Aryrsulfatase A missense mutations G86D, Y201C, D255H, and E212D causing Metachromatic Leukodystrophy. Am J Med Genet 2000;91:68-73
8. Baum H, Dogson KS, Spencer B. The assay of the arylsulfatase A and B in human urine. Clin Chim Acta 1959;4:453-455
9. Gieselmann V, Schmidt B, Von Figura K. In vitro mutagenesis of potencial N-glycosylation sites of arylsulfatase A. J Biol Chem 1992;267:13262-13266
10. Schierau A, Dietz F, Lange H , et al. Interaction of arylsulfatase A with UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase. J Biol Chem 1999;274:3651-3658
11. Marcão A, Amaral O, Pinto E, et al. Metachromatic Leucodystrophy in Portugal – Finding of four new molecular lesions: C300F, P425T, g.1190-1191insC, and g.2408delC. Hum Mutat 1999;13:337-338(mutation in Brief #232)
12. Marcao A, Simonis H, Schestag F, Sa Miranda MC, Gieselmann V. Biochemical characterization of two (C300F, P425T) arylsulfatase a missense mutations. Am J Med Genet 2003;116A(3):238-42
13. Chang PL, Ameen M, Yu CZ, Kelly BM. Effect of Ammonium Chloride on subcellular distribution of lysosomal enzymes in human fibroblasts. Exp Cell Res 1988;176:258-267
14. Perusi C, Gomez-Lira M, Duyff RF, et al. Mutations associated with very late onset metachromatic leukodystrophy. Clin Genet 1999;55:130
15. Tylky-Szymanska A, Berger J, Loschl B, et al. Late juvenile metachromatic leukodystrophy (MLD) in three patients with a similar clinical course and identical mutation on one allele. Clin Genet 1996;50:287-292

16. Halsall DJ, Halligan EP, Elsey TS, Cox TM. Metachromatic leucodystrophy: a newly identified mutation in arylsulfatase A, D281Y, found as a compound heterozygote with I179L in an adult onset case. Hum Mutat 1999;14:447
17. Baumann N, Turpin JC, Lefevre M, Colsch B. Motor and psycho-cognitive clinical types in adult metachromatic leukodystrophy: genotype/phenotype relationships? J Physiol Paris 2002;96(3-4):301-6
18. Comabella M, Waye JS, Raguer N, et al. Late-Onset metachromatic leukodystrophy clinically presenting as isolated peripheral neuropathy: Compound heterozygosity for the IVS2+1G>A mutation and a newly identified missense mutation (Thr408Ile) in a Spanish family. Ann Neurol 2001;50:108-112
19. Fressinaud C, Vallat JM, Masson M, et al. Adult onset metachromatic leukodystrophy presenting as isolated peripheral neuropathy. Neurology 1992;42:1396-1398
20. Felice KJ, Gomez-Lira M, Natowicz M, et al. Adult-onset metachromatic leukodystrophy: a gene mutation with isolated polyneuropathy. Neurology 2000;55:1036-1039
21. Coulter-Mackie MB, Applegarth DA, Toone JR, Gagnier L, Anzarut AR, Hendson G. Isolated peripheral neuropathy in atypical metachromatic leukodystrophy: a recurrent mutation. Can J Neurol Sci 2002;29(2):159-63
22. Baumann N, Masson M, Carreau V, et al. Adult forms of metachromatic leucodystrophy: Clinical and biochemical approach. Dev Neurosci 1991;3:211-215
23. Hageman AT, Gabreels FJ, de Jong JG, et al. Clinical symptoms of adult metachromatic leucodystrophy and arylsulfatase A pseudodeficiency. Arch Neurol 1995;52:408-413.
24. Cengiz N, Ozbenli T, Onar M, Yildiz L, Ertas B. Adult metachromatic leukodystrophy: three cases with normal nerve conduction velocities in a family. Acta Neurol Scand 2002;105(6):454-7
25. Brion S, Mikol J, Graveleau. Leucodystrophie metachromatique de l àdulte jeune. Etude clinique, biologique et ultrastructurale. Rev Neurol 1970;122:161-176
26. Kreysing J, Bohne W, Bosenberg C, et al. High Residual Arylsulfatase A (ARSA) Activity in a Patient with Late-Infantile Metachromatic Leukodystrophy. Am J Hum Genet 1993;53:339-346
27. Arbour TL, Silver K, Hechtman P, et al. Variable onset of metachromatic leukodystrophy in a Vietnamese family. Pediatr Neurol 2000;23:173-176
28. Von Figura K, Steckel F, Hasilik A. Juvenile and adult metachromatic leukodystrophy: Partial restauration of arylsulfatase A (cerebroside sulfatase) activity by inhibitors of thiol proteinases. Proc Natl Acad Sci 1983;80:6066-6070
29. Von Bülow R, Schmidt B, Dierks T, et al. Defective oligomerization of arylsulfatase A as a cause of its instability in lysosomes and metachromatic leukodystrophy. J Biol Chem 2002;277:9455-9461

# 2B. IMPACT OF ARSA MISSENSE MUTATIONS IN THE ENZYME OLIGOMERIZATION CAPACITY

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# Oligomerization capacity of two arylsulfatase A mutants: C300F and P425T

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**Abstract**

Arylsulfatase A (ARSA) is a lysosomal enzyme implicated in most cases of metachromatic leukodystrophy (MLD). The quaternary structure of ARSA is pH-dependent: at neutral pH, ARSA is a homodimeric protein; at lysosomal (acidic) pH, ARSA is homo-octameric. This dimer–octamer transition seems to be of major importance for the stability of the enzyme in the lysosomal milieu. Sedimentation analysis was used to study the oligomerization capacity of C300F and P425T-substituted ARSA, two MLD-associated forms of the enzyme displaying reduced lysosomal half-lives. P425T-ARSA displays a modest reduction in its octamerization capacity. In contrast, the C300F mutation strongly interferes with the octamerization process of ARSA but not with its dimerization capacity. Interestingly, a major fraction of dimeric ARSA-C300F is composed of covalently linked ARSA molecules, through a thiol-cleavable bond that probably involves Cys414 residues from each monomer. Our data support the notion that the reduced lysosomal half-life of some mutated forms of ARSA is related to deficient octamerization.
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*Keywords:* Arylsulfatase A; Metachromatic leukodystrophy; Protein stability; Lysosome

Arylsulfatase A (ARSA) is a lysosomal enzyme involved in the catabolism of various sulfate-containing substrates. The major physiologic substrate of ARSA is 3-*O*-sulfogalactosyl ceramide (sulfatide) which accounts for 3.5–4% of the total lipids of myelin.

ARSA has been crystallized and its three-dimensional structure has been solved. The core of the enzyme consists of two β-pleated sheets, linked together by several hydrogen bonds and one disulfide bridge (Cys300–Cys414). The large central β-pleated sheet is decorated on both sides by several helices such that the enzyme has high structural resemblance to bacterial alkaline phosphatase [1].

The quaternary structure of ARSA is highly pH dependent. The enzyme oscillates between two states: dimeric and octameric. At neutral pH the dimeric form predominates; at the lysosomal acidic pH the enzyme exists mainly as a homo-octamer, composed of four dimers arranged in a ring-like structure. This pH-dependent dimer–octamer equilibrium is regulated by deprotonation–protonation of Glu424, whereas the non-covalent dimerization involves other regions of the enzyme and is not pH dependent [1].

Mutations in the ARSA gene are, by far, the most frequent cause of metachromatic leukodystrophy (MLD), a lysosomal storage disorder with autosomal recessive inheritance. The intralysosomal accumulation of sulfatide occurs mainly in oligodendrocytes, Schwann cells and neurons, and to a lesser extent in visceral organs like kidney, gallbladder, and liver [2].

Presently, more than 90 different disease-causing mutations in the ARSA gene are known (HumanGeneMutationDatabase). For many of these mutations a clear relationship between the observed genetic alteration and the enzymatic deficiency can be easily established. This is the case for some non-sense mutations or for site-splicing mutations that strongly

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interfere with the production of a normal mRNA [3–5]. However, in the case of missense mutations this task requires extensive biochemical experiments, despite the fact that the three-dimensional structure of ARSA is already known [1]. A recent report on the properties of ARSA-P426L, a mutated ARSA form displaying a normal enzymatic specific activity, illustrates this point [6]. X-ray analysis of crystallized ARSA-P426L failed to reveal any major structural alteration. However, ARSA-P426L is a very unstable enzyme in lysosomes. A complete biochemical characterization of this protein provided the answer to this problem: the P426L mutation negatively interferes with the octamerization of ARSA, a process that must occur in order to yield a protease-resistant enzyme capable of surviving in the hostile lysosomal compartment. Thus, even a subtle conformational alteration in the structure of ARSA may produce a dramatic effect on its in vivo stability.

In a previous work we described two MLD-associated mutations in the ARSA gene [7]. The first mutation, ARSA-C300F, was found in a homozygotic state in a child displaying a severe phenotype of MLD. The second mutation, ARSA-P425T, was found in a juvenile patient, in heterozygosity with one of the most frequent mutations in MLD, the P426L mutation [4]. More recently, we demonstrated that ARSA-C300F displays an almost null specific enzymatic activity whereas ARSA-P425T was found to have 12% of the activity of the normal enzyme. Data suggesting that both mutated forms are correctly targeted to the lysosomal compartment were also provided. However, pulse-chase analysis revealed that both mutated enzymes are rapidly degraded in the lysosome [8].

As an attempt to understand the reduced half-lives of these two ARSA mutant forms, we have analyzed their homopolymerization capacity. Our results indicate that ARSA-P425T displays a reduction in its octamerization capacity. The octamerization capacity of ARSA-C300F is highly reduced. Interestingly, ARSA-C300F is able to form dimers implying that formation of the Cys300–Cys414 disulfide bond is not a prerequisite for the dimerization process of the enzyme. Furthermore, a major fraction of dimeric ARSA-C300F is composed of covalently linked ARSA molecules, through a thiol-cleavable covalent bond that probably involves opposing Cys414 residues from each monomer. It is hypothesized that the structural alterations occurring at the monomer–monomer interface in the ARSA-C300F enzyme change the association angle of the two monomers impairing the octamerization process.

These data lend support to the notion that a deficiency in the octamerization process of ARSA may be the reason behind the reduced lysosomal half-lives of some mutated forms of this enzyme.

## Materials and methods

*Materials.* Cell culture media, antibiotics, and fetal calf serum were from Invitrogen Life Technologies. Mammalian protease inhibitor cocktail was from Sigma. Centricon-10 concentrators were from Amicon. The High Molecular Weigh Calibration Kit was from Amersham–Pharmacia Biotech. Other reagents were from Sigma.

*Arylsulfatase A activity determination.* ARSA activity determination was done using the artificial ARSA substrate *p*-nitrocatechol sulfate [9].

*Sample preparation.* Stably transfected Ltk$^-$ cell lines expressing C300F-ARSA, P425T-ARSA, and WT-ARSA have been previously generated [8]. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 5% fetal calf serum. Sixteen confluent 75 cm$^2$ flasks of Ltk$^-$ cells expressing C300F-ARSA, P425T-ARSA, or WT-ARSA were harvested. The cells were sonicated in 0.02% (w/v) Triton X-100 with 520 μM AEBSF, 0.4 μM aprotinin, 10.5 μM leupeptin, 18 μM bestatin, 7.5 μM pepstatin A, 7 μM E-64, 50 μM iodoacetamide, 10 μM PMSF, and 1 mM EDTA. The suspensions were first clarified by a 20 min centrifugation at 15,000*g* and the supernatants were then subjected to a 20 min ultracentrifugation at 145,000*g* using a T-1270 rotor (Sorvall). The cleared supernatants were concentrated fourfold using Centricon-10 concentrators from Amicon.

*Sedimentation analysis.* Cell extracts containing 240 mU WT-ARSA activity or, in the case of the mutants, an equivalent amount of ARSA protein as quantified by Western blotting analysis (see below), were diluted with appropriated buffer (10 mM Tris–HCl, 150 mM NaCl (pH 7.0) or 100 mM NaAc/HAc, and 150 mM NaCl (pH 4.8 or pH 5.2)) to a final volume of 500 μl. The samples were then applied onto the top of discontinuous sucrose gradients (1.8 ml of 7.5%, 1.8 ml of 10%, 1.7 ml of 15%, 1.6 ml of 21%, 1.5 ml of 25%, and 1.4 ml of 30% (w/v) sucrose in the buffer used to prepare the corresponding sample). After centrifugation at 165,000*g* for 16 h, at 4 °C, in a TST 41.14 swing-out rotor (Sorvall), 12 fractions of 0.86 ml were collected from the bottom of the tube.

The High Molecular Weigh Calibration Kit from Amersham–Pharmacia Biotech was used to calibrate the gradients.

*SDS–PAGE and Western blot.* Proteins present in each gradient fraction were precipitated with 10% (w/v) trichloroacetic acid. After 30 min on ice, the precipitated protein was pelleted (15 min at 15,000*g*), washed with acetone, solubilized in Laemmli sample buffer [10], and analyzed by SDS–PAGE and Western blotting using an ARSA antiserum, raised against recombinant ARSA in rabbits. SDS–PAGE was performed in 1.0 mm thick, 10% polyacrylamide gels using the Laemmli discontinuous buffer system [10].

## Results and discussion

Sedimentation analysis was used to compare the oligomerization capacity of C300F and P425T-ARSA mutants with WT-ARSA. As shown in Fig. 1, at pH 4.8 about 75% of WT-ARSA is found in fractions 4–6 corresponding to an apparent molecular mass of c.a. 500 kDa. This value is in good agreement with a molecular mass of about 496 kDa, the theoretical value expected for the ARSA octamer. About 20% of ARSA is found in fractions 8–10 (estimated molecular mass of 120 kDa c.a.), indicating that dimeric WT-ARSA (calculated molecular mass of 124 kDa) is also present under these experimental conditions. At pH 5.2, the amount of wild type ARSA in the octameric form decreases to about 60% of total (Fig. 1A, fractions 4–6)

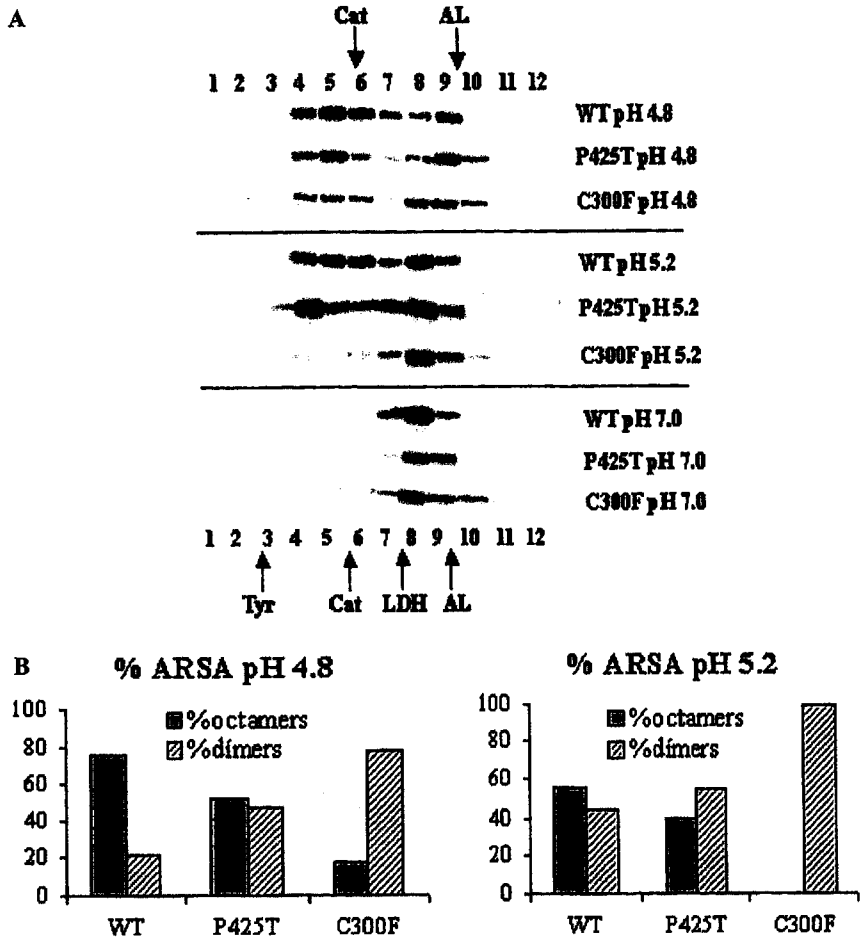


Fig. 1. Oligomerization of WT-ARSA, C300F-ARSA, and P425T-ARSA at different pH. WT, C300F, and P425T-ARSA were subjected to sucrose gradient centrifugation at pH 4.8, pH 5.2, and pH 7.0. After fractionation of the gradients (1–12 fractions from bottom to top) proteins were analyzed by SDS–PAGE and Western blot. Native molecular mass markers used to calibrate the gradient (pH 7.0): thyroglobulin (Tyr, fraction 3, 669 kDa), catalase (Cat, fraction 6, 232 kDa), lactate dehydrogenase (LDH, fraction 8, 140 kDa), and bovine serum albumin (AL, fractions 9 and 10, 67 kDa). At pH 4.8, the thyroglobulin and the lactate dehydrogenase precipitated, but catalase and bovine serum albumin maintained their localization. The results presented in the graphics result from the quantification of Western blot signals by laser scan densitometry of X-ray films. At pH 4.8, we considered dimers at fractions 8–10 and octamers at fractions 4–6. At pH 5.2, we considered dimers at fractions 7–9 and octamers at fractions 4–6, with the exception of P425T-ARSA octamers that we considered at fractions 3–5.

while the amount in the dimeric form increases to about 40% (Fig. 1A, fractions 7–9). At pH 7.0, the vast majority of the wild type enzyme is detected as a dimer (fractions 7–9, Fig. 1) as expected [1].

When the sedimentation properties of ARSA-P425T were analyzed, a different behavior was observed. At pH 4.8, the amount of ARSA-P425T in the octameric state is clearly lower than the one observed for the normal enzyme (see Fig. 1B). At pH 5.2, this difference is still detected, although not so markedly, suggesting that the P425T substitution not only shifts the pH at which half of ARSA exists as a octamer towards lower values (c.a. pH 4.8), but also the profile of the dimer–octamer equilibrium as a function of pH. Although no attempts were made to quantify these two variables, the data obtained for ARSA-P425T are very similar to the results described recently for the P426L mutation [6]. No effects on the dimerization capacity of ARSA-P425T were noted in these experiments (see Fig. 1, panel "pH 7.0").

In contrast with the results described above, the C300F mutation was found to have a dramatic effect on the octamerization process of the enzyme. Indeed, as shown in Fig. 1, only a small fraction of ARSA-C300F behaves as an octameric protein at pH 4.8. At pH 5.2, octameric ARSA-C300F is no longer detected (Figs. 1A and B). These data indicate that the C300F mutation induces conformational alterations in ARSA such that the octamerization process of the enzyme is highly affected.

As mentioned above, Cys300 forms an intramolecular disulfide bridge with Cys414. These two cysteines localize at the monomer–monomer interface (and not at the dimer–dimer interface). Thus, the observation that C300F mutation interferes with the octamerization process of ARSA would be easy to understand if dimerization of ARSA-C300F was impaired. Strikingly, this is clearly not the case. As shown in Fig. 1, the majority of ARSA-C300F behaves as a dimeric protein upon sedimentation analysis at all the pHs tested. How can this result be explained? Perhaps the easiest explanation would be to

assume that the C300F substitution results in a distortion of the monomer–monomer interface changing the association angle of the two molecules. Such dimers could still interact with other dimers but a stable ring of four dimers would be difficult to obtain. Although this hypothesis remains speculative, data showing that indeed some structural alterations at the monomer–monomer interface are introduced by the C300F mutation were obtained when the behavior of ARSA was analyzed by non-reducing SDS–PAGE. As shown in Fig. 2, when WT-ARSA is subjected to SDS–PAGE only a single band (apparent molecular mass of 62 kDa) corresponding to the monomeric protein is observed, as expected. In contrast, ARSA-C300F displays a dual behavior: a fraction of the enzyme co-migrates with the WT-ARSA; the other fraction of ARSA-C300F migrates as a 124 kDa protein. Under reducing conditions this 124 kDa protein band disappears with the concomitant increase of the 62 kDa protein band (monomeric ARSA-C300F; see lane 4). These data, together with the observation that only one protein (corresponding to ARSA) is immunoprecipitated from $^{35}$S-labeled Ltk$^-$ cells expressing ARSA-C300F by an anti-ARSA antibody [8], indicate that the 124 kDa species represents a dimer of ARSA in which the two monomers are linked together by at least one disulfide bridge.

The three-dimensional structure of WT-ARSA reveals that from the 14 cysteines present in the molecule only Cys38 and Cys294 are in a reduced state. In ARSA-C300F, an additional reduced cysteine would be expected: Cys414. Although all these three cysteines are located at the monomer–monomer interface, it is plausible to assume that the structure of Cys414 will be the most affected by the C300F substitution. The presence

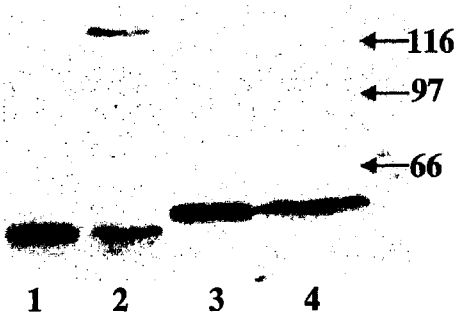


Fig. 2. Non-reducing SDS–PAGE analysis of the C300F-ARSA mutant. Proteins in homogenates of Ltk$^-$ cells, stably expressing WT (lanes 1 and 3; 80 μg protein/lane) and C300F-ARSA (lanes 2 and 4; 200 μg protein/lane), were resolved by SDS–PAGE, under reducing or non-reducing conditions, and analyzed by Western blot. Lanes 1 and 2—the homogenates were incubated with 20 mM IAA, for 10 min, at room temperature and with Laemmli sample buffer, without DTT, for 5 min at 90 °C, before being applied on the SDS–PAGE gel. Lanes 3 and 4—the homogenates were incubated with 20 mM IAA for 10 min, at room temperature, and with 100 mM DTT and Laemmli sample buffer for 5 min at 90 °C, before being applied on the SDS–PAGE gel. The numbers in the right indicate the molecular masses of the applied standards in kDa.

of the hydrophobic and bulky side chain of a phenylalanine at this position could not only induce a reorientation of the sulfide group of Cys414 towards the exterior of the monomer but also bring in close proximity, at the monomer–monomer interface, the sulfide groups of Cys414 from each monomer.

The aim of the work presented here was to address the relationship between the reduced in vivo half-life of some MLD-associated ARSA mutant forms and their oligomerization capacities. As suggested recently [6], mutations in the ARSA protein impairing its pH-dependent octamerization result in unstable enzymes rapidly degraded by lysosomal cathepsins. We selected for this study two mutated forms of the enzyme: ARSA-P425T and ARSA-C300F, both displaying a reduced half-life [8]. The first mutation involves an amino acid residue adjacent to Glu424, the proposed molecular switch controlling the pH-dependent dimer–octamer transition of ARSA [1]. Thus, considering the short half-life of ARSA-P425T, a deficient octamerization capacity for this mutated ARSA form would be expected, as described recently for ARSA-P426L [6]. Indeed, such phenomenon was observed—although ARSA-P425T is able to octamerize, the efficiency of this process is clearly lower than the one displayed by the normal enzyme.

The second mutation characterized in this work involves the substitution of cysteine 300 by a phenylalanine. In the normal enzyme, Cys300 lies at the monomer–monomer interface establishing an intramolecular disulfide bridge with Cys414. Thus, a priori, some effect (if any) on the dimerization process of this mutated ARSA enzyme would be expected. Strikingly, the octamerization (and not the dimerization) process of ARSA-C300F is highly impaired, a property that probably reflects the existence of an altered association angle of the two ARSA monomers. As described for the ARSA-P426L mutated form [6], it is possible that this incapacity of ARSA-C300F in forming an octameric structure results in an increased accessibility to the lysosomal proteases.

In summary, the data presented here suggest that an impairment in the octamerization process of ARSA may be the reason behind the lysosomal instability of some mutated ARSA forms.

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## References

[1] G. Lukatela, N. Krauss, K. Theis, T. Selmer, V. Gieselmann, K. von Figura, Saenger, Crystal structure of human arylsulfatase A:

the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis, Biochemistry 37 (1998) 3654–3664.

[2] K. von Figura, V. Gieselmann, J. Jaeken, Metachromatic leukodystrophy, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Basis of Inherited Disease, eigth ed., McGraw-Hill, New York, 2001, pp. 3695–3724.

[3] A.L. Fluharty, C.B. Fluharty, W. Bohne, K. von Figura, V. Gieselmann, Two new arylsulfatase A (ARSA) mutations in a juvenile metachromatic leukodystrophy (MLD) patient, Am. J. Hum. Genet. 49 (1991) 1340–1350.

[4] A. Polten, A.L. Fluharty, C.B. Fluharty, J. Kappler, K. von Figura, V. Gieselmann, Molecular basis of different forms of metachromatic leukodystrophy, N. Engl. J. Med. 324 (1991) 18–22.

[5] N.M. Pastor-Soler, M.A. Rafi, J.D. Hoffman, D. Hu, D.A. Wenger, Metachromatic leukodystrophy in the Navajo Indian population: a splice site mutation in intron 4 of the arylsulfatase A gene, Hum. Mutat. 4 (1994) 199–207.

[6] R. von Bülow, B. Schmidt, T. Dierks, N. Schwabauer, K. Schilling, E. Weber, I. Usón, K. von Figura, Defective oligomerization of arylsulfatase A as a cause of its instability in lysosomes and metachromatic leukodystrophy, J. Biol. Chem. 277 (2002) 9455–9461.

[7] A. Marcão, O. Amaral, E. Pinto, R. Pinto, M.C. Sá Miranda, Metachromatic leucodystrophy in Portugal-finding of four new molecular lesions: C300F, P425T, g.1190-1191insC, and g.2408delC (mutations in brief no. 232, online), Hum. Mutat. 13 (1999) 337–338.

[8] A. Marcão, H. Simonis, F. Schestag, M.C. Sá Miranda, V. Gieselmann, Biochemical characterization of two (C300F, P425T) arylsulfatase a missense mutations, Am. J. Med. Genet. 116 (2003) 238–242.

[9] H. Baum, K.S. Dogsan, B. Spencer, The assay of arylsulfatase A and B in urine, Clin. Chim. Acta 4 (1959) 453–455.

[10] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

# CHAPTER 3. GENERAL CONCLUSIONS

# GENERAL CONCLUSIONS

## A. Genetic epidemiology of ARSA deficiencies

### 1. Metachromatic Leukodystrophy

MLD is a severe lysosomal storage disorder due to deficient activity of the enzyme Arylsulfatase A. The human ARSA gene has been cloned and characterised already in 1990 (Kreysing *et al.*, 1990), and more than ninety MLD-causing mutations have been so far described in different populations (von Figura *et al.*, 2001 and *www.hgmd.org*). Nevertheless, regarding the genetic epidemiology of this pathology in Portugal, nothing was known before this work. The results presented here, concerning the molecular characterization of the Portuguese MLD patients, are thus an important contribution to the understanding of the genetic epidemiology of this disease.

Comparing our results with those obtained for other European populations (Chapter 1 – Section 1A.2, Figure 1) we observed that the most prominent differences are the higher frequency of the ARSA null mutation IVS2+1G>A, which represents 60% of the MLD alleles among unrelated Portuguese patients, and the lower frequency of mutation P426L, which represents only 3% of those alleles. Both these mutations have been reported to have a frequency of 25% each among European MLD patients (Polten *et al.*, 1991). The highest frequency of IVS2+1G>A mutation reported in Europe was around 40% and was found in the British population (Barth *et al.*, 1993a). Mutation P426L also was not found among the Spanish population (Gort *et al.*, 1999).

In spite of the high frequency of mutation IVS2+1G>A in Portugal, our results also illustrate the molecular heterogeneity that is reported to be associated with MLD: eight different mutations (c412insC, IVS2+1G>A, I179S, D255H, C300F, c1040delC,

P425T, P426L) were found among the sixteen families studied, three of them (C300F, c1040delC, P425T) were never reported in non-Portuguese patients. Portuguese thus seem to have a unique mutation profile regarding MLD, and this first molecular characterisation of Portuguese families will facilitate the posterior study of other MLD Portuguese patients.

The study of the haplotypes associated with specific genetic alterations can be important for the characterisation of a mutational event, thus contributing to the understanding of the general aspects of the occurrence of genetic alterations, but can also provide some interesting clues regarding the movements of the populations. Based on the linkage disequilibria between mutation IVS2+1G>A and a single rare haplotype, this mutation was proposed to have an ancient single European origin (Zlotogora *et al.*, 1994a). Its high frequency in different regions of Portugal, together with its appearance in linkage disequilibria with the same single rare haplotype, seems to indicate that this is a very ancient mutation in our population. The Portuguese could even have contributed to its spread among other populations, at the time of the Crusades, as was speculated by Zlotogora *et al.*, (1994a).

Haplotyping has also been done for the other MLD mutant alleles and our results were in agreement with the fixed haplotypes previously proposed for mutations P426L, I179S and D255H (Coulter-Mackie *et al.*, 1997b). In spite of the small number of unrelated cases reported, the possible existence of common ancestors is suggested.

## 2. Arylsulfatase A-pseudodeficiency

Arylsulfatase A-pseudodeficiency is a relatively frequent but poorly understood condition, which is characterised by a reduction of ARSA normal activity, but is not associated with a MLD clinical phenotype (von Figura *et al.*, 2001). The genetic polymorphisms in the basis of ARSA-PD were identified (Gieselmann *et al.*, 1989) and their frequency determined in different populations (von Figura *et al.*, 2001). The interference of this condition in the biochemical diagnosis of MLD is well known (von Figura *et al.*, 2001), but once again nothing was known regarding its epidemiology in Portugal.

Approximately eighteen percent of the Portuguese are carriers of one of the three alleles known to be associated with the ARSA-PD: 9,3% have the 2417G/ 3352G allele while 8,7% have the 2417G/ 3352A allele. The 2417A/ 3352G allele was found in only one individual. Taking the frequencies of the three alleles altogether, this is the highest value reported in a European population, which demonstrates the importance of this condition in Portugal.

The haplotype analysis of ARSA-PD alleles in different populations has long raised several hypotheses regarding the order of appearance of the alterations that characterise the ARSA-PD alleles and its appearance as a single or a recurrent event (Zlotogora *et al.*, 1994a, Ott *et al.*, 1997). The determination of the haplotype associated with ARSA-PD and non-ARSA-PD alleles was also done in this work.

Seventeen different haplotypes were identified in non-ARSA-PD alleles, whereas the ARSA-PD allele 2417G/ 3352G was found to be exclusively associated to one of the rare haplotypes: Bgl I (T) Bsr I (C) Bam HI (G) Nla III$^{2}$ (G) Nla III$^{1}$ (G) Aci I (G), which was found in 8,6% of the normal alleles and in none of the ARSA-PD alleles

2417G/ 3352A. In contrast, the ARSA-PD alleles 2417G/ 3352A and 2417A/ 3352G were found associated to different haplotypes.

These results seem to confirm the association of the ARSA-PD allele 2417G/ 3352G with a highly conserved haplotype (Zlotogora *et al.*, 1994a) which together with its high frequency in Europeans and its absence in non-Europeans (Ott *et al.*, 1997), strongly suggests a single ancient origin posterior to the divergence of European and Asian populations.

On the other hand, the identification of the ARSA-PD alleles 2417G/ 3352A and 2417A/ 3352G in different haplotypes does not allow us to exclude the possible existence of de novo mutations on different haplotype backgrounds nor the possible divergence of the polymorphic backgrounds of the mutated alleles.

**B. Structural/ functional relationships of ARSA**

ARSA has been extensively studied and its three-dimensional structure is already known (Lukatela *et al.*, 1998). Nevertheless, a clear relationship between an observed genetic alteration in the ARSA gene and the consequent enzymatic deficiency is not always easy to establish, and in the case of missense mutations can require extensive biochemical experiments and reveal unsuspected results.

Three novel missense mutations were biochemically characterised in this work: mutations C300F and P425T, found in two Portuguese families, and mutation F219V, found in one patient of Tamil origin. These mutations were found to be associated with different clinical forms of MLD: C300F mutation was found in homozygosity in one late infantile patient; mutation P425T was found in one juvenile patient; and mutation F219V was found in homozygosity in one adult patient.

Unexpectedly, the F219V mutation was found to cause a severe reduction of ARSA activity, below the levels that would be expected to an MLD adult patient with such a very late onset. This reduced activity was determined to be due to low stability of the mutant enzyme and to a reduced specific activity. Despite the fact that its sorting is affected by $NH_4Cl$, which indicates that at least part of it is able to escape from the ER, its indirect structural analysis based on its recognition by several monoclonal antibodies revealed that it might have a severe structural alteration.

Regarding the mutations found in the Portuguese patients, no enzyme activity could be expressed from C300F allele, while about 10% residual enzyme activity could be expressed from P425T allele. Biosynthesis studies revealed that both mutant enzymes are unstable and their increased cellular secretion upon addition of $NH_4Cl$ suggests that, at least part of these enzymes is able to reach the Golgi compartment. This is also supported by the presence of mannose 6-phosphate residues on the oligosaccharide side chains of the mutant enzymes.

The relationship between the reduced in vivo half-live of both these mutants and their oligomerization capacities was also addressed and our data support the notion that the reduced lysosomal half-life of some mutated forms of ARSA might be related to deficient octamerization.

Mutation P425T affects an amino acid residue adjacent to Glu424 that is proposed to be responsible for the pH-dependent dimer-octamer transition of ARSA (Lukatela *et al.*, 1998). Therefore, the deficient octamerization capacity of this mutant could be expected, as described for other ARSA mutants with short half-live (von Bülow *et al.*, 2002).

Mutation C300F affects an amino acid residue that lies at the ARSA monomer-monomer interface establishing an intramolecular disulfide bridge with Cys414. Thus, some effect on the dimerization process of this mutated ARSA enzyme could be expected.

Our results indicate that ARSA-P425T displays a reduction in its octamerization capacity, while the C300F mutation strongly interferes with the octamerization process of ARSA but not with its dimerization capacity. This implies that formation of the Cys300-Cys414 disulfide bond is not a prerequisite for the dimerization process of the enzyme. We could also observe that a major fraction of dimeric ARSA-C300F is composed of covalently linked ARSA molecules, through a thiol-cleavable bond that probably involves opposing Cys414 residues from each monomer. This observation allowed us to speculate that the structural alterations occurring at the monomer-monomer interface in the ARSA-C300F enzyme change the association angle of the two monomers impairing the octamerization process.

As described for the ARSA-P426L mutated form (von Bülow *et al.*, 2002), it is thus possible that the incapacity of ARSA-C300F and ARSA-P425T in forming an octameric structure results in an increased accessibility to the lysosomal proteases, which could explain their low stability.

## C. Genotype-phenotype correlation in MLD

The existence or not of a genotype-phenotype correlation in "monogenic" disorders can be important for the prediction of the age of onset and clinical progression of the disease in each individual, but is also important to the understanding of the general mechanisms of the pathology. In some cases, the non-existence of a genotype-

phenotype correlation can provide important clues regarding the importance of other genetic or environmental factors.

It is usually accepted that a general genotype-phenotype correlation exists in MLD and that the clinical heterogeneity observed in this pathology is due to different amounts of residual enzyme activity. In general, patients with two null mutations present the late infantile form, while the presence of at least one mutation leading to some residual activity mitigates the course of the disease leading to juvenile or adult forms (von Figura *et al.*, 2001). Once again our results corroborate this general rule: the late infantile patients here analysed have two alleles codifying enzymes with null activity (IVS2+1G>A/ IVS2+1G>A, IVS2+1G>A/ c412insC, IVS2+1G>A/ D255H, c1040delC/ c1040delC, C300F/ C300F), while the late-onset patients (juvenile and adult forms) have one or two alleles codifying enzymes with some residual activity (IVS2+1G>A/ I179S, P425T/ P426L).

In spite of this general correlation, the prediction of the onset and evolution of the disease based on the genotype is not straightforward and some clinical heterogeneity can be observed among individuals with the same genotype, even within the same family, and especially in the case of the adult patients. This may be related with their longer life expectancy, which allows the effect of other environmental or genetic factors to become more evident.

In fact, some inter and intrafamilial heterogeneity, regarding the age of onset and clinical evolution, could be observed among the adult MLD patients discussed here, which have the same genotype (IVS2+1G>A/ I179S). Some biochemical heterogeneity could also be observed regarding the elevated sulfatide excretion in urine, which was present only in one of these families. In these specific cases, the presence of mutation

I179S, which originates a protein with some residual enzyme activity and that is known to be particularly susceptible to proteolytic degradation (Fluharty *et al.*, 1991), can also contribute to this heterogeneity. The different amount of lysosomal proteases in each individual could explain this variability. Juvenile and adult patients have been reported with this mutation. Some of the adult patients are compound heterozygotes with a null mutation (Fluharty *et al.*, 1991; Berger *et al.*, 1997).

In spite of the observed variability, the recently suggested association of mutation I179S with early psychiatric symptoms and intellectual impairment (Baumann *et al.*, 2002) could also be observed in our patients.

Other examples also raise some doubts regarding the genotype-phenotype correlation in MLD. In our results this is illustrated by the identification of mutation F219V, which revealed to be associated with a severe reduction of ARSA activity, in a 37 years old woman that presented exclusively severe cognitive and intellectual impairment. Furthermore, this F219V-ARSA seems to be partially retained in the ER and might have a severe structural alteration.

On the other hand, at least one patient has been described presenting high residual enzyme activities but an early onset of the disease (Kreysing *et al.*, 1993). Both these examples illustrate a lack of correlation between the biochemical and the clinical phenotype that remains unexplained, and they seem once again to indicate that in spite of the genotype-phenotype correlation usually accepted to exist in MLD, other factors, genetic or environmental, might affect the disease's onset and progression.

## D. Implication of ARSA-PD in the pathogenesis of non-MLD related pathologies

The high frequency of the ARSA-PD associated alleles among the general population (von Figura *et al.*, 2001) and their almost constant combined frequency in different populations (Ott *et al.*, 1997), might suggest an advantage for the carriers of ARSA-PD alleles. Nevertheless, to date there are no plausible hypotheses for this advantage.

On the other hand, the possibility that ARSA-PD is implicated in the pathogenesis of other non-MLD associated pathologies could not also be excluded, in spite of the large number of studies that have been done on this subject (von Figura et al., 2001).

One strong possibility that remains to be proved is the higher susceptibility of individuals with ARSA-PD to develop psychiatric alterations. The fact that some MLD adult patients present early clinical signs that lead to the misdiagnosis of schizophrenia make the schizophrenic patients an interesting candidate group to test ARSA-PD.

The determination of the frequency of the ARSA-PD associated alleles among a group of Portuguese patients with schizophrenia revealed a statistically significant higher frequency of allele 2417G/ 3352A. If this result is confirmed in a larger sample, this can reveal to be an important contribution to the elucidation of the clinical implications of ARSA-PD, although the mechanism on the basis of this association will remain to be elucidated.

# CHAPTER 4. FUTURE PROSPECTS

## FUTURE PROSPECTS

### 1. Importance of specific ARSA amino acid residues to the structure and function of the enzyme

ARSA has been intensively studied, not only to try to understand the cellular pathogenic mechanisms of MLD, but also to try to understand the normal cellular mechanisms of processing, targeting and activation of lysosomal soluble hydrolases and sulfatases. Its three dimensional structure was solved (Lukatela *et al.*, 1998) and putative amino acid residues important for its structure and function were identified (Lukatela *et al.*, 1998; Waldow *et al.*, 1999). Some of this knowledge resulted from the biochemical characterisation of mutations that were found in MLD patients (von Bülow *et al.*, 2002).

In spite of all these studies, in most cases of novel missense mutations is still not possible to predict the effect of the mutation on the structure and function of the enzyme. Thus, it is still important to continue characterising the novel mutations, not only in a perspective of a better understanding of the normal cellular mechanisms responsible for the enzyme's acquisition of normal structure and function, but also to try to understand the abnormal cellular processes associated with each mutation, which can be important to design therapeutic strategies.

### 2. Additional genetic or environmental factors affecting ARSA activity "in vivo"

Cases have been reported that raise some doubts regarding the existence of a straightforward genotype-phenotype correlation in MLD, or that at least raise the hypothesis of the existence of other genetic or environmental factors that influence the activity of ARSA in vivo (Alves *et al.*, 1986; Clark *et al.*, 1989; Polten *et al.*, 1991).

Our own results seem to corroborate this hypothesis. The detection and further investigation of such cases may contribute to the identification of these factors, which can be important not only for the understanding of the pathogenesis of MLD but also to predict the age of onset and clinical evolution of the disease in each case. This can be also important to delineate different therapeutic approaches.

Two possible factors that could modulate the enzyme's activity in vivo are the efficiency of the ARSA activator protein Saposin B, which could be affected by some genetic polymorphisms, and the pattern of lysosomal proteases characteristic of each patient, whose efficiency could also be affected by genetic polymorphisms at the level of the different proteases.

It is also known that some of the mutants are retained at the ER level, and even if they have some residual activity they are not able to get in the lysosome. The different efficiency of the cellular machinery at the quality control level in the ER could also contribute to some variability.

These factors, whose understanding can also contribute to the development of therapeutic strategies, are especially important when the mutated enzymes have some residual enzyme activity, which is frequently the case of the late-onset patients.

### 3. Implication of ARSA-PD in the pathogenesis of pathologies other than MLD

An increased susceptibility of individuals with ARSA-PD to develop neuropsychiatric symptoms as been long hypothesised, although never proved. Our results seem to indicate the association between ARSA-PD and schizophrenia. However, the base for this association was not elucidated.

The GABA receptors have been implicated in the pathogenesis of some of the psychiatric conditions speculated to be associated with ARSA-PD, including schizophrenia (Arnold *et al.*, 1999; Kittler *et al.*, 2002). Additionally, sulfatide was reported to be implicated in the binding of GABA to its receptors (Chweh *et al.*, 1983), although the nature of this implication and its physiologic significance are unknown, and at least one subtype of GABA receptors is associated with lipid raft microdomains (Becher *et al.*, 2001). Recent reports also established the importance of sulfatide in the appropriate localization and maintenance of ion channels clusters (Ishibashi *et al.*, 2002).

It could thus be interesting to analyse the correct formation, localization, stability and activity of the different GABA receptor subtypes. The generation of a transgenic mouse with low ARSA activity could be important not only to do this kind of studies, but also to verify the development of neurologic symptoms.

**4. New therapeutic approaches**

Several reports have recently been made regarding a possible new therapeutic strategy that uses chemical chaperones to stabilize mutant enzymes with some residual activity, but that are missfolded and thus retained in the ER and degraded through the proteasome. The activity of beta-glucosidase, alpha-galactosidase A and beta-galactosidase, three lysosomal enzymes whose absence causes Gaucher Disease, Fabry Disease and GM1-gangliosidosis, respectively, could be enhanced in patients' cultured cells using inhibitors of these enzymes in subinhibitory intracellular concentrations (Fan *et al.*, 1999; Asano *et al.*, 2000; Tominaga *et al.*, 2001; Sawkar *et al.*, 2002).

The biochemical characterization of several ARSA mutants revealed that some of them are completely or partially retained in the ER, and thus even if they have some residual enzyme activity they are not able to get in the lysosome (Harvey *et al.*, 1993; Kappler *et al.*, 1994; Hess *et al.*, 1996a; Hermann *et al.*, 2000). In the cases of ER retention of a mutant with some residual enzyme activity, this kind of therapeutic approach may prove important, especially because there are no other proved alternatives for therapy in MLD. However, until today there are no such chemical chaperones reported to act on ARSA stabilization.

Several ARSA mutants, usually associated with late-onset MLD, revealed to have some enzyme activity and to be able to reach the lysosome, but are rapidly degraded by the lysosomal proteases (Polten *et al.*, 1991). Some of these mutants can be stabilized by inhibition of lysosomal proteases (von Figura *et al.*, 1983), and thus the development of therapeutic strategies to stabilize this kind of mutants that are able to reach the lysosome and that have residual enzyme activity, but that are extremely sensitive to lysosomal proteases and rapidly degraded, could also be interesting.

# REFERENCES

## REFERENCES

Abe A, Arend LJ, Lee L, Lingwood C, Brady RO, Shayman JA. Glycosphingolipid depletion in fabry disease lymphoblasts with potent inhibitors of glucosylceramide synthase. Kidney Int. (2000) 57(2): 446-54.

Ahn VE, Faull KF, Whitelegge JP, Fluharty AL, Prive GG. Crystal structure of saposin B reveals a dimeric shell for lipid binding. Proc Natl Acad Sci U S A (2003a) 100(1): 38-43.

Ahn VE, Faull KF, Whitelegge JP, Higginson J, Fluharty AL, Prive GG. Expression, purification, crystallization, and preliminary X-ray analysis of recombinant human saposin B. Protein Expr Purif (2003b) 27(1): 186-93.

Alves D, Pires MM, Guimaraes A, Miranda MC. Four cases of late onset metachromatic leucodystrophy in a family: clinical, biochemical and neuropathological studies. J Neurol Neurosurg Psychiatry (1986) 49(12): 1417-22.

Ameen M, Lazzarino DA, Kelly BM, Gabel CA, Chang PL. Deficient glycosylation of arylsulfatase A in pseudo arylsulfatase-A deficiency. Mol Cell Biochem (1990) 92(2): 117-27.

Arbour LT, Silver K, Hechtman P, Treacy EP, Coulter-Mackie MB. Variable onset of metachromatic leukodystrophy in a Vietnamese family. Pediatr Neurol (2000) 23(2): 173-6.

Arnold SE. Schizophrenia. in: Koliatsos VE and Ratan RR (Eds.), Cell Death and Diseases of the Nervous System, Humana Press, Totowa, New Jersey, 1999, pp. 527-541.

Arvanitis D, Dumas M, Szuchet S. Myelin palingenesis. 2. Immunocytochemical localization of myelin/oligodendrocyte glycolipids in multilamellar structures. Dev Neurosci (1992) 14(5-6): 328-35.

Asano N, Ishii S, Kizu H, Ikeda K, Yasuda K, Kato A, Martin OR, Fan JQ. In vitro inhibition and intracellular enhancement of lysosomal alpha-galactosidase A activity in Fabry lymphoblasts by 1-deoxygalactonojirimycin and its derivatives. Eur J Biochem (2000) 267(13):4179-86.

Ballabio A., Shapiro LJ. Steroid sulfatase deficiency and X-linked ichthyosis. In: Scriver CR, et al, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill, 2001:3695-3724.

Bardosi A, Friede RL, Ropte S, Goebel HH. A morphometric study on sural nerves in metachromatic leucodystrophy. Brain (1987) 110 ( Pt 3): 683-94.

Barth ML, Fensom A, Harris A. Prevalence of common mutations in the arylsulphatase A gene in metachromatic leukodystrophy patients diagnosed in Britain. Hum Genet (1993a) 91(1): 73-7.

Barth ML, Fensom A, Harris A. Missense mutations in the arylsulphatase A genes of metachromatic leukodystrophy patients. Hum Mol Genet (1993b) 2(12): 2117-21.

Barth M.L., Ward C., Harris A., Saad A., Fensom A., Frequency of Arylsulfatase A pseudodeficiency associated mutations in a healthy population, J. Med. Genet. (1994) 31 (9): 667-671.

Barth ML, Fensom A, Harris A. Identification of seven novel mutations associated with metachromatic leukodystrophy. Hum Mutat (1995) 6(2): 170-6.

Baum H, Dogsan KS, Spencer B. The assay of arylsulfatase A and B in urine. Clin Chim Acta (1959) 4: 453-455.

Baumann N, Turpin JC, Lefevre M, Colsch B. Motor and psycho-cognitive clinical types in adult metachromatic leukodystrophy: genotype/phenotype relationships? J Physiol Paris (2002) 96(3-4): 301-6.

Becher A, White JH, McIlhinney RA. The gamma-aminobutyric acid receptor B, but not the metabotropic glutamate receptor type-1, associates with lipid rafts in the rat cerebellum. J Neurochem (2001) 79(4):787-95.

Benjamins JA, Guarnieri M, Miller K, Sonneborn M, McKhann GM. Sulphatide synthesis in isolated oligodendroglial and neuronal cells. J Neurochem (1974) 23(4): 751-7.

Berger J, Gmach M, Fae I, Molzer B, Bernheimer H. A new polymorphism of arysulfatase A within the coding region. Hum Genet (1996) 98(3): 348-50.

Berger J, Loschl B, Bernheimer H, Lugowska A, Tylki-Szymanska A, Gieselmann V, Molzer B. Occurrence, distribution, and phenotype of arylsulfatase A mutations in patients with metachromatic leukodystrophy. Am J Med Genet (1997) 69(3): 335-40.

Berger J, Gmach M, Mayr U, Molzer B, Bernheimer H. Coincidence of two novel arylsulfatase A alleles and mutation 459+1G>A within a family with metachromatic leukodystrophy: molecular basis of phenotypic heterogeneity. Hum Mutat (1999) 13(1): 61-8.

Beutler E. Enzyme replacement therapy for Gaucher's disease. Baillieres Clin Haematol (1997) 10(4): 751-63.

Bognar SK, Furac I, Kubat M, Cosovic C, Demarin V. Croatian population data for arylsulfatase a pseudodeficiency-associated mutations in healthy subjects, and in patients with Alzheimer-type dementia and Down syndrome. Arch Med Res (2002) 33 (5): 473-7.

Bohne W, von Figura K, Gieselmann V. An 11-bp deletion in the arylsulfatase A gene of a patient with late infantile metachromatic leukodystrophy. Hum Genet (1991) 87(2): 155-8.

Bond CS, Clements PR, Ashby SJ, Collyer CA, Harrop SJ, Hopwood JJ, Guss JM. Structure of a human Lysosomal Sulfatase. Structure (1997) 5(2): 227-89.

Bosio A, Binczek E, Stoffel W. Functional breakdown of the lipid bilayer of the myelin membrane in central and peripheral nervous system by disrupted galactocerebroside synthesis. Proc Natl Acad Sci U S A. (1996) 93(23): 13280-5.

Braulke T. Origin of lysosomal proteins in: Subcellular Biochemistry, Volume 27: Biology of the Lysosome, Lloyd and Mason eds., Plenum Press, New York, 1996.

Brennan MJ, Hannah JH, Leininger E. Adhesion of Bordetella pertussis to sulfatides and to the GalNAc beta 4Gal sequence found in glycosphingolipids. J Biol Chem (1991) 266(28): 18827-31.

Bruns GA, Mintz BJ, Leary AC, Regina VM, Gerald PS. Expression of human arylsufatase-A in man-hamster somatic cell hybrids. Cytogenet Cell Genet (1978) 22(1-6): 182-5.

Buschard K, Hoy M, Bokvist K, Olsen HL, Madsbad S, Fredman P, Gromada J. Sulfatide controls insulin secretion by modulation of ATP-sensitive K(+)-channel activity and Ca(2+)-dependent exocytosis in rat pancreatic beta-cells. Diabetes (2002) 51(8): 2514-21.

Cerami C, Kwakye-Berko F, Nussenzweig V. Binding of malarial circumsporozoite protein to sulfatides [Gal(3-SO4)beta 1-Cer] and cholesterol-3-sulfate and its dependence on disulfide bond formation between cysteines in region II. Mol Biochem Parasitol (1992) 54(1): 1-12.

Chang PL, Davidson RG. Pseudo arylsulfatase-A deficiency in healthy individuals: genetic and biochemical relationship to metachromatic leukodystrophy. Proc Natl Acad Sci U S A (1983) 80(23): 7323-7.

Chang PL, Rosa NE, Varey PA, Kihara H, Kolodny EH, Davidson RG. Diagnosis of pseudo-arylsulfatase A deficiency with electrophoretic techniques. Pediatr Res (1984) 18(10): 1042-5.

Christomanou H, Sandhoff K. A sensitive fluorescence assay for the simultaneous and separate determination of arylsulphatases A and B. Clin Chim Acta (1977) 79(3): 527-31.

Chweh AY, Leslie SW. Enhancement by sulfatide of Na+-independent [3H]GABA binding in mouse brain. Biochem Biophys Res Commun (1983) 112(3):827-32.

Clarke JT, Skomorowski MA, Chang PL. Marked clinical difference between two sibs affected with juvenile metachromatic leukodystrophy.Am J Med Genet (1989) 33(1): 10-3.

Coetzee T, Fujita N, Dupree J, Shi R, Blight A, Suzuki K, Suzuki K, Popko B. Myelination in the absence of galactocerebroside and sulfatide: normal structure with abnormal function and regional instability. Cell (1996) 86(2): 209-19.

Consiglio A, Quattrini A, Martino S, Bensadoun JC, Dolcetta D, Trojani A, Benaglia G, Marchesini S, Cestari V, Oliverio A, Bordignon C, Naldini L. In vivo gene therapy of metachromatic leukodystrophy by lentiviral vectors: correction of neuropathology and protection against learning impairments in affected mice. Nat Med (2001) 7(3): 310-16.

Conzelmann E, Sandhoff K. Partial Enzyme Deficiencies: Residual Activities and the Development of Neurological Disorders. Dev Neurosci (1983/84) 6: 58-71.

Cosma MP, Pepe S, Annunziata I, Newbold RF, Grompe M, Parenti G, Ballabio A. The multiple sulfatase deficiency gene encodes an essential and limiting factor for the activity of sulfatases. Cell (2003) 113(4): 445-56.

Coulter-Mackie MB, Gagnier L, Beis MJ, Applegarth DA, Cole DE, Gordon K, Ludman MD. Metachromatic leucodystrophy in three families from Nova Scotia, Canada: a recurring mutation in the arylsulphatase A gene. J Med Genet (1997a) 34(6): 493-8.

Coulter-Mackie M, Gagnier L. Two new polymorphisms in the arylsulfatase A gene and their haplotype associations with normal, metachromatic leukodystrophy and pseudodeficiency alleles. Am J Med Genet (1997b) 73(1): 32-5.

Coulter-Mackie MB, Gagnier L. Two novel mutations in the arylsulfatase A gene associated with juvenile (R390Q) and adult onset (H397Y) metachromatic leukodystrophy. Hum Mutat (1998 ) Suppl 1: S254-6.

Devaiah AK, Raife TJ, Barton JA, Olson JD. Inhibition of human platelet function by sulfatides. Blood Coagul Fibrinolysis (2000) 11(6): 543-50.

D'Hooge R, Hartmann D, Manil J, Colin F, Gieselmann V, De Deyn P. Neuromotor alterations and cerebellar deficits in aged arylsulfatase A-deficient transgenic mice. Neuroscience Letters (1999a) 273(2): 93-96.

D'Hooge R, Coenen R, Gieselmann V, Lüllmann-Rauch R, De Deyn P. Decline in brainstem auditory-evoked potentials coincides with loss of spiral ganglion cells in arylsulfatase A-deficient mice. Brain Res (1999b) 352-356.

D'Hooge R, Van Dam D, Franck F, Gieselmann V, De Deyn P. Hyperactivity, neuromotor defects, and impaired learning and memory in a mouse model for metachromatic leukodystrophy. Brain Res (2001) 35-43.

Dierks T, Schmidt B, Von Figura K. Conversion of cysteine to formylglycine: a protein modification in the endoplasmic reticulum. Proc Natl Acad Sci USA (1997) 94(22): 11963-8.

Dierks T, Miech C, Hummerjohann J, Schmidt B, Kertesz MA, Von Figura K. Posttranslational formation of formylglycine in prokaryotic sulfatases by modification of either cysteine or serine. J Biol Chem (1998) 273(40): 25560-4.

Dierks T, Lecca MR, Schlotterhose P, Schmidt B, von Figura K. Sequence determinants directing conversion of cysteine to formylglycine in eukaryotic sulfatases. EMBO J (1999) 18(8): 2084-91.

Dierks T, Schmidt B, Borissenko LV, Peng J, Preusser A, Mariappan M, von Figura K. Multiple sulfatase deficiency is caused by mutations in the gene encoding the human C(alpha)-formylglycine generating enzyme. Cell (2003) 113(4): 435-44.

Ding Z, Issekutz TB, Downey GP, Waddell TK. L-selectin stimulation enhances functional expression of surface CXCR4 in lymphocytes: implications for cellular activation during adhesion and migration. Blood (2003) 101(11): 4245-52.

Draghia R, Letourneur F, Drugan C, Manicom J, Blanchot C, Kahn A, Poenaru L, Caillaud C. Metachromatic leukodystrophy: identification of the first deletion in exon 1 and of nine novel point mutations in the arylsulfatase A gene. Hum Mutat (1997) 9(3): 234-42.

Emre S, Topcu M, Terzioglu M, Renda Y. Arylsulfatase A pseudodeficiency incidence in Turkey. Turk J Pediatr (2000) 42(2): 115-7.

Eto Y, Tahara T, Koda N, Yamaguchi S. Prenatal diagnosis of metachromatic leukodystrophy: a diagnosis with amniotic fluid by DEAE-Sepharose column chromatography and its confirmation by kidney lipid analysis. J Inherit Metab Dis (1982) 5(2): 77-8.

Eto Y, Kawame H, Hasegawa Y, Ohashi T, Ida H, Tokoro T. Molecular characteristics in Japanese patients with lipidosis: novel mutations in metachromatic leukodystrophy and Gaucher disease. Mol Cell Biochem (1993) 119(1-2): 179-84.

Fan JQ, Ishii S, Asano N, Suzuki Y. Accelerated transport and maturation of lysosomal alpha-galactosidase A in Fabry lymphoblasts by an enzyme inhibitor. Nat Med (1999) 5(1): 112-5.

Farrell DF, McKhann GM. Characterization of cerebroside sulfotransferase from rat brain. J Biol Chem (1971) 246(15): 4694-702.

Farrell DF. Heterozygote detection in MLD. Allelic mutations at the ARA locus. Hum Genet (1981) 59(2): 129-34.

Ferret-Sena V, Sena A, Besnard F, Fressinaud C, Rebel G, Sarlieve LL. Comparison of the mechanisms of action of insulin and triiodothyronine on the synthesis of cerebroside sulfotransferase in cultures of cells dissociated from brains of embryonic mice. Dev Neurosci (1990) 12(2): 89-105.

Fey J, Balleininger M, Borissenko LV, Schmidt B, Von Figura K, Dierks T. Characterization of posttranslational formylglycine formation by luminal components of the endoplasmic reticulum. J Biol Chem (2001) 276(50): 47021-8.

Fluharty AL, Stevens RL, Kihara H. Cerebroside sulfate hydrolysis by fibroblasts from a parent with metachromatic leukodystrophy. J Pediatr (1978) 92(5): 782-4.

Fluharty AL, Fluharty CB, Bohne W, Von Figura K, Gieselmann V. Two new arylsulfatase A (ARSA) mutations in a juvenile metachromatic leukodystrophy (MLD) patient. Am J Hum Genet (1991) 49: 1340-1350.

Franco B, Meroni G, Parenti G, Levilliers J, Bernard L, Gebbia M, Cox L, Maroteaux P, Sheffield L, Rappold GA et al. A cluster of sulfatase genes on Xp22.3: mutations in chondrodysplasia punctata (CDPX) and implications for warfarin embryopathy. Cell (1995) 81(1): 15-25.

Gieselmann V, Polten A, Kreysing J, Von Figura K, Arylsulfatase A pseudodeficiency: loss of a polyadenylylation signal and a N-glycosylation site. Proc Natl Acad Sci USA (1989) 86(23): 9436-9440.

Gieselmann V, Polten A, Kreysing J, Kappler J, Fluharty A, Von Figura K. Molecular genetics of metachromatic leukodystrophy. Dev Neurosci (1991) 13(4-5): 222-7. Review.

Gieselmann V, Schmidt B, Von Figura K. In vitro mutagenesis of potencial N-glycosylation sites of arylsulfatase A. J Biol Chem (1992) 267: 13262-13266.

Gieselmann V, Zlotogora J, Harris A, Wenger D, Morris CF. Molecular genetics of metachromatic leucodystrophy. Hum Mutat (1994) 4: 233-242.

Gieselmann V, Matzner U, Hess B, Lullmann-Rauch R, Coenen R, Hartmann D, D'Hooge R, DeDeyn P, Nagels G. Metachromatic leukodystrophy: molecular genetics and an animal model. J Inherit Metab Dis (1998) 21(5): 564-74. Review.

Goebel HH, Busch H. Abnormal lipopigments and lysosomal residual bodies in metachromatic leukodystrophy. Adv Exp Med Biol (1989) 266: 299-309.

Gomez-Lira M, Perusi C, Mottes M, Pignatti PF, Manfredi M, Rizzuto N, Salviati A. Molecular genetic characterization of two metachromatic leukodystrophy patients who carry the T799G mutation and show different phenotypes; description of a novel null-type mutation. Hum Genet (1998) 102(4): 459-63.

Gort L, Coll MJ, Chabas A. Identification of 12 novel mutations and two new polymorphisms in the arylsulphatase A gene: haplotype and genotype-phenotype correlation studies in Spanish metachromatic leukodystrophy patients. Hum Mutat (1999) 14(3): 240-248.

Graljaard H. Sulfatide lipidosis or metachromatic leukodystrophy. In: Genetic Metabolic Diseases. Amsterdam, Elsevier/ North-Holland Biomedical Press, 1980, 215-236.

Guibaud P, Garcia I, Guyonnet CL. La detection de l'heterozygotisme pour la sulfatidase. Lyon Med (1973) 229: 1215.

Gustavson KH, Hagberg B. The incidence and genetics of metachromatic leucodystrophy in northern Sweden. Acta Paediatr Scand (1971) 60(5): 585-90.

Hagberg B, Sourander P, Svennerholm L. Sulfatide lipidosis in childhood. Report of a case investigated during life and at autopsy. Am J Dis Child (1962) 104: 644-56.

Halsall DJ, Halligan EP, Elsey TS, Cox TM. Metachromatic leucodystrophy: a newly identified mutation in arylsulphatase A, D281Y, found as a compound heterozygote with I179L in an adult onset case. Hum Mutat (1999) 14(5): 447.

Harzer K, Kustermann-Kuhn B. Brain galactolipid content in a patient with pseudoarylsulfatase A deficiency and coincidental diffuse disseminated sclerosis, and in patients with metachromatic, adreno-, and other leukodystrophies. J Neurochem (1987) 48(1): 62-6.

Harvey JS, Nelson PV, Carey WF, Robertson EF, Morris CP. An arylsulfatase A (ARSA) missense mutation (T274M) causing late-infantile metachromatic leukodystrophy. Hum Mutat (1993) 2(4): 261-7.

Harvey JS, Carey WF, Morris CP. Importance of the glycosylation and polyadenylation variants in metachromatic leukodystrophy pseudodeficiency phenotype. Hum Mol Genet (1998) 7(8): 1215-9.

Hasegawa Y, Kawame H, Eto Y. Mutations in the arylsulfatase A gene of Japanese patients with metachromatic leukodystrophy. DNA Cell Biol (1993) 12(6): 493-8.

Hasegawa Y, Kawame H, Ida H, Ohashi T, Eto Y. Single exon mutation in arylsulfatase A gene has two effects: loss of enzyme activity and aberrant splicing. Hum Genet (1994) 93(4): 415-20.

Hasilik A, Von Figura K, Conzelmann E, Nehrkorn H, Sandhoff K. Lysosomal enzyme precursors in human fibroblasts. Activation of cathepsin D precursor in vitro and activity of beta-hexosaminidase A precursor towards ganglioside GM2. Eur J Biochem (1982) 125(2): 317-21.

Heim P, Claussen M, Hoffmann B, Conzelmann E, Gartner J, Hartzer K, Hunnemen DH, Kohler W, Kurlemann G, Kohlschutter A. Leukodystrophy incidence in Germany. Am J Med Genet (1997) 71(4): 475-8.

Heinisch U, Zlotogora J, Kafert S, Gieselmann V. Multiple mutations are responsible for the high frequency of metachromatic leukodystrophy in a small geographic area. Am J Hum Genet (1995) 56(1): 51-7.

Hermann S, Schestag F, Polten A, Kafert S, Penzien J, Zlotogora J, Baumann N, Gieselmann V. Characterization of four arylsulfatase A missense mutations G86D, Y201C, D255H, and E312D causing metachromatic leukodystrophy. Am J Med Genet (2000) 91(1): 68-73.

Herz B, Bach G. Arylsulfatase A in pseudodeficiency. Hum Genet (1984) 66(2-3): 147-50.

Hess B, Kafert S, Heinisch U, Wenger DA, Zlotogora J, Gieselmann V. Characterization of two arylsulfatase A missense mutations Asp335>Val and Thr274>Met causing late infantile metachromatic leukodystrophy. Hum Mutat (1996a) 7: 311-317.

Hess B, Saftig P, Hartmann D, Coenen R, Lüllmann-Rauch R, Goebel HH, Evers M, Von Figura K, D'Hooge R, Nagels G, De Deyn P, Peters C, Gieselmann V. Phenotype of arylsulfatase A-deficient mice: relationship to human metachromatic leukodystrophy. Proc Natl Acad Sci USA (1996b) 93: 14821-14826.

Honke K, Kobayashi T, Fujii T, Gasa S, Xu M, Takamaru Y, Kondo R, Tsuji S, Makita A. An adult-type metachromatic leukodystrophy caused by substitution of serine for glycine-122 in arylsulfatase A. Hum Genet (1993) 92(5): 451-6.

Honke K, Hirahara Y, Dupree J, Suzuki K, Popko B, Fukushima K, Fukushima J, Nagasawa T, Yoshida N, Wada Y, Taniguchi N. Paranodal junction formation and spermatogenesis require sulfoglycolipids. Proc Natl Acad Sci USA (2002) 99(7): 4227-32.

Hopwood JJ, Ballabio A. Multiple Sulfatase Deficiency and the Nature of the Sulfatase family. In: Scriver CR, et al, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill, (2001): 3725-3732.

Humbel R. Rapid method for measuring arylsulfatase A and B in leucocytes as a diagnosis for sulfatidosis, mucosulfatidosis and mucopolysaccharidosis VI. Clin Chim Acta. (1976) 68(3): 339-41.

Ishibashi T, Dupree JL, Ikenaka K, Hirahara Y, Honke K, Peles E, Popko B, Suzuki K, Nishino H, Baba H. A myelin galactolipid, sulfatide, is essential for maintenance of ion channels on myelinated axon but not essential for initial cluster formation. J Neurosci (2002) 22(15): 6507-14.

Ishizuka I, Tadano K, Nagata N, Niimura Y, Nagai Y. Hormone-specific responses and biosynthesis of sulfolipids in cell lines derived from mammalian kidney. Biochim Biophys Acta (1978) 541(4): 467-82.

Jeyakumar M, Butters TD, Cortina-Borja M, Hunnam V, Proia RL, Perry VH, Dwek RA, Platt FM. Delayed symptom onset and increased life expectancy in Sandhoff disease mice treated with N-butyldeoxynojirimycin. Proc Natl Acad Sci U S A. (1999) 96(11): 6388-93.

Kafert S, Heinisch U, Zlotogora J, Gieselmann V. A missense mutation P136L in the arylsulfatase A gene causes instability and loss of activity of the mutant enzyme. Hum Genet (1995) 95(2): 201-4.

Kakkis ED. Enzyme replacement therapy for the mucopolysaccharide storage disorders. Expert Opin Investig Drugs. (2002) 11(5): 675-85.

Kappler J, von Figura K, Gieselmann V. Late-onset metachromatic leukodystrophy: molecular pathology in two siblings. Ann Neurol (1992) 31(3): 256-61.

Kappler J, Sommerlade HJ, von Figura K, Gieselmann V. Complex arylsulfatase A alleles causing metachromatic leukodystrophy. Hum Mutat (1994) 4(2): 119-27.

Kihara H, Ho CK, Fluharty AL, Tsay KK, Hartlage PL. Prenatal diagnosis of metachromatic leukodystrophy in a family with pseudo arylsulfatase A deficiency by the cerebroside sulfate loading test. Pediatr Res (1980) 14(3): 224-7.

Kittler JT, McAinsh K, Moss SJ. Mechanisms of GABAA receptor assembly and trafficking: implications for modulation of inhibitory neurotransmission. Mol Neurobiol (2002) 26(2-3): 251-68.

Knaust A., Schmidt B., Dierks T., von Bulow R., von Figuras K. Residues critical for formylglycine formation and/or catalytic activity of arylsulfatase A. Biochemistry. (1998) Oct 6;37(40):13941-6.

Kondo R, Wakamatsu N, Yoshino H, Fukuhara N, Miyatake T, Tsuji S. Identification of a mutation in the arylsulfatase A gene of a patient with adult-type metachromatic leukodystrophy. Am J Hum Genet (1991) 48(5): 971-8.

Kornblatt MJ, Knapp A, Levine M, Schachter H, Murray RK. Studies on the structure and formation during spermatogenesis of the sulfoglycerogalactolipid of rat testis. Can J Biochem (1974) 52(8): 689-97.

Kornfeld S, Mellman I. The biogenesis of lysosomes. Annu Rev Cell Biol (1989) 5: 483-525.

Kornfeld S, Sly WS. I-Cell Disease and Pseudo-Hurler Polydystrophy: Disorders of Lysosomal Enzyme Phosphorylation and Localization. In: Scriver CR, et al, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill, 2001:3695-3724.

Kreysing J, Von Figura K, Gieselmann V. Structure of the Arylsulfatase A gene. Eur J Biochem (1990) 191(3): 627-631.

Kreysing J, Bohne W, Bosenberg C, Marchesini S, Turpin JC, Baumann N, von Figura K, Gieselmann V. High residual arylsulfatase A (ARSA) activity in a patient with late-infantile metachromatic leukodystrophy. Am J Hum Genet (1993) 53(2): 339-46.

Krivan HC, Olson LD, Barile MF, Ginsburg V, Roberts DD. Adhesion of Mycoplasma pneumoniae to sulfated glycolipids and inhibition by dextran sulfate. J Biol Chem (1989) 264(16): 9283-8.

Krivit W, Sung JH, Shapiro EG, Lockman LA. Microglia: the effector cell for reconstitution of the central nervous system following bone marrow transplantation for lysosomal and peroxisomal storage diseases. Cell Transplant (1995) 4(4): 385-92. Review.

Krivit W, Peters C, Shapiro EG. Bone marrow transplantation as effective treatment of central nervous system disease in globoid cell leukodystrophy, metachromatic leukodystrophy, adrenoleukodystrophy, mannosidosis, fucosidosis, aspartylglucosaminuria, Hurler, Maroteaux-Lamy, and Sly syndromes, and Gaucher disease type III. Curr Opin Neurol (1999) 12(2): 167-76. Review.

Kurosawa K, Ida H, Eto Y. Prevalence of arylsulphatase A mutations in 11 Japanese patients with metachromatic leukodystrophy: identification of two novel mutations. J Inherit Metab Dis (1998) 21(7): 781-2.

Lee L, Abe A, Shayman JA. Improved inhibitors of glucosylceramide synthase. J Biol Chem. (1999) 274(21): 14662-9.

Lee-Vaupel M, Conzelmann E. Assay for cerebroside sulfate (sulfatide) sulfatase in cultured skin fibroblasts with the natural activator protein. Clin Chim Acta (1987) 168(1): 55-68.

Leinekugel P, Michel S, Conzelmann E, Sandhoff K. Quantitative correlation between the residual activity of β-hexosaminidase A and arylsulfatase A and the severity of the resulting lysosomal storage disease. Hum Genet (1992) 88: 513-520.

Leistner S, Young E, Meaney C, Winchester B. Pseudodeficiency of Arylsulfatase A: strategy for clarification of genotype in families of subjects with low ASA activity and neurological symptoms. J Inherit Metab Dis (1995) 18(6): 710-716.

Leroy JG, Van Elsen AF, Martin JJ, Dumon JE, Hulet AE, Okada S, Navarro C. Infantile metachromatic leukodystrophy. Confirmation of a prenatal diagnosis. N Engl J Med (1973) 288(26): 1365-9.

Li ZG, Waye JS, Chang PL. Diagnosis of arylsulfatase A deficiency. Am J Med Genet (1992) 43(6): 976-82.

Lissens W, Vervoort R, Van Regemorter N, Van Bogaert P, Freund M, Verellen-Dumoulin C, Seneca S, Liebaers I. A D255H substitution in the arylsulphatase A gene of two unrelated Belgian patients with late-infantile metachromatic leukodystrophy. J Inherit Metab Dis (1996) 19(6): 782-6.

Lloyd JB. The taxonomy of Lysosomes and related structures in: Subcellular Biochemistry, Volume 27: Biology of the Lysosome, Lloyd and Mason eds., Plenum Press, New York, 1996.

Lugowska A, Czartoryska B, Tylki-Szymanska A, Bisko M, Zimowski JG, Berger J, Molzer B. Prevalence of arylsulfatase A pseudodeficiency allele in metachromatic leukodystrophy patients from Poland. Eur Neurol (2000) 44(2): 104-7.

Lukatela G, Krauss N, Theis K, Selmer T, Gieselmann V, Von Figura K, Saenger W. Crystal structure of human arylsulfatase A: the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis. Biochemistry (1998) 37(11): 3654-3664.

Luyten JA, Wenink PW, Steenbergen-Spanjers GC, Wevers RA, Ploos van Amstel HK, de Jong JG, van den Heuvel LP. Metachromatic leukodystrophy: a 12-bp deletion in exon 2 of the arylsulfatase A gene in a late infantile variant. Hum Genet (1995) 96(3): 357-60.

Malde AD, Naik LD, Pantvaidya SH, Oak SN. An unusual presentation in a patient with metachromatic leukodystrophy. Anaesthesia (1997) 52(7): 690-4.

Malone MJ, Stoffyn P. A comparative study of brain and kidney glycolipids in metachromatic leucodystrophy. J Neurochem (1966) 13(11): 1037-45.

Marcus J, Dupree JL, Popko B. Myelin-associated glycoprotein and myelin galactolipids stabilize developing axo-glial interactions. J Cell Biol (2002a) 156(3): 567-77.

Marcus J, Popko B. Galactolipids are molecular determinants of myelin development and axo-glial organization. Biochim Biophys Acta (2002b) 1573(3): 406-13.

Martensson E. Sulfatides of human kidney. Isolation, identification, and fatty acid composition. Biochim Biophys Acta (1966) 116(3): 521-31.

Martin JJ, Ceuterick C, Mercelis R, Joris C. Pathology of peripheral nerves in metachromatic leucodystrophy. A comparative study of ten cases. J Neurol Sci (1982) 53(1): 95-112.

Matzner U, Harzer K, Learish RD, Barranger JA, Gieselmann V. Long-term expression and transfer of arylsulfatase A into brain of arylsulfatase A-deficient mice transplanted with bone marrow expressing the arylsulfatase A cDNA from a retroviral vector. Gene Ther (2000) 7(14): 1250-1257.

Matzner U, Hartmann D, Lullmann-Rauch R, Coenen R, Rothert F, Mansson JE, Fredman P, D'Hooge R, De Deyn PP, Gieselmann V. Bone marrow stem cell-based gene transfer in a mouse model for

metachromatic leukodystrophy: effect on visceral and nervous system disease manifestations. Gene Ther (2002) 9(1): 53-56.

Meikle PJ, Hopwood JJ, Clague AE, Carey WF: Prevalence of lysosomal storage disorders. JAMA. (1999) 281(3): 249-54.

Mehl E, Jatzkewitz H. Evidence For The Genetic Block In Metachromatic Leucodystrophy (Ml). Biochem Biophys Res Commun (1965) 19: 407-11.

Merten M, Thiagarajan P. Role for sulfatides in platelet aggregation. Circulation (2001) 104(24): 2955-60.

Miech C, Dierks T, Selmer T, Von Figura K, Schmidt B. Arylsulfatase from Klebsiella pneumonie carries a formyglycine generated from a serine. J Biol Chem (1998) 273(9): 4835-7.

Molzer B, Sundt-Heller R, Kainz-Korschinsky M, Zobel M. Elevated sulfatide excretion in heterozygotes of metachromatic leukodystrophy: dependence on reduction of arylsulfatase A activity. Am J Med Genet (1992) 44(4): 523-6.

Muller HM, Reckmann I, Hollingdale MR, Bujard H, Robson KJ, Crisanti A. Thrombospondin related anonymous protein (TRAP) of Plasmodium falciparum binds specifically to sulfated glycoconjugates and to HepG2 hepatoma cells suggesting a role for this molecule in sporozoite invasion of hepatocytes. EMBO J (1993) 12(7): 2881-9.

Muschol N, Matzner U, Tiede S, Gieselmann V, Ullrich K, Raulke T. Secretion of phosphomannosyl – deficient arylsufatase A and cathepsin D from isolated human macrophages. Biochem J (2002) 368(pt 3): 845-53.

Natomi H, Sugano K, Takaku F, Iwamori M. Glycosphingolipid composition of the gastric mucosa. A role of sulfatides in gastrointestinal mucosal defense? J Clin Gastroenterol (1990) 12 Suppl 1: S52-7.

Nelson PV, Carey WF, Morris CP. Population frequency of the arylsulphatase A pseudo-deficiency allele. Hum Genet (1991) 87(1): 87-8.

Neufeld EF. Lysosomal storage diseases. Annu Rev Biochem (1991) 60: 257-80. Review.

Neufeld EF and Muenzer J. The mucopolysaccharidoses. In: Scriver CR, et al, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill, 2001:3695-3724.

Norton WT, Poduslo SE. Myelination in rat brain: changes in myelin composition during brain maturation. J Neurochem (1973) 21(4): 759-73.

Norton WR, Poduslo SE: Biochemical studies of metachromatic leukodystrophy in three siblings. Acta Neuropathol (1982) 57: 188.

Olson LD, Gilbert AA. Characteristics of Mycoplasma hominis adhesion. J Bacteriol (1993) 175(10): 3224-7.

Osterbye T, Jorgensen KH, Fredman P, Tranum-Jensen J, Kaas A, Brange J, Whittingham JL, Buschard K. Sulfatide promotes the folding of proinsulin, preserves insulin crystals, and mediates its monomerization. Glycobiology (2001) 11(6): 473-9.

Ott R, Waye JS, Chang PL. Evolutionary origins of two tightly linked mutations in arylsulphatase-A pseudodeficiency. Hum Genet (1997) 101(2): 135-40.

Parenti G, Meroni G, Ballabio A. The sulfatase gene family. Curr Opin Genet Dev (1997) 7(3): 386-91. Review.

Park DS, Poretz RD, Stein S, Nora R, Manowitz P. Association of Alcoholism with the N-Glycosilation Polymorphism of Pseudodeficient Human Arylsulfatase A. Alcohol Clin Exp Res (1996) 20(2): 228-233.

Pastor-Soler NM, Rafi MA, Hoffman JD, Hu D, Wenger DA. Metachromatic leukodystrophy in the Navajo Indian population: a splice site mutation in intron 4 of the arylsulfatase A gene. Hum Mutat (1994) 4(3): 199-207.

Pastor-Soler NM, Schertz EM, Rafi MA, de Gala G, Wenger DA. Metachromatic leukodystrophy among southern Alaskan Eskimos: molecular and genetic studies. J Inherit Metab Dis (1995) 18(3): 326-32.

Pedron CG, Gaspar PA, Giugliani R, Pereira ML. Arylsulfatase A pseudodeficiency in healthy Brazilian individuals. Braz J Med Biol Res (1999) 32(8): 941-5.

Penzien JM, Kappler J, Herschkowitz N, Schuknecht B, Leinekugel P, Propping P, Tonnesen T, Lou H, Moser H, Zierz S, et al. Compound heterozygosity for metachromatic leukodystrophy and arylsulfatase A pseudodeficiency alleles is not associated with progressive neurological disease. Am J Hum Genet (1993) 52(3): 557-64.

Phelan MC, Thomas GR, Saul RA, Rogers RC, Taylor HA, Wenger DA, McDermid HE. Cytogenetic, biochemical, and molecular analysis of a 22q 13 deletion. Am J Med Genet (1992) 43(5): 872-6.

Platt FM, Neises GR, Dwek RA, Butters TD. N-butyldeoxynojirimycin is a novel inhibitor of glycolipid biosynthesis. J Biol Chem (1994) 269(11): 8362-5.

Platt FM, Neises GR, Reinkensmeier G, Townsend MJ, Perry VH, Proia RL, Winchester B, Dwek RA, Butters TD. Prevention of lysosomal storage in Tay-Sachs mice treated with N-butyldeoxynojirimycin. Science (1997) 276(5311): 428-31.

Platt FM, Jeyakumar M, Andersson U, Priestman DA, Dwek RA, Butters TD, Cox TM, Lachmann RH, Hollak C, Aerts JM, Van Weely S, Hrebicek M, Moyses C, Gow I, Elstein D, Zimran A. Inhibition of substrate synthesis as a strategy for glycolipid lysosomal storage disease therapy. J Inherit Metab Dis (2001) 24(2): 275-90.

Polten A, Fluharty AL, Fluharty CB, Kappler J, Von Figura K, Gieselmann V. Molecular basis of different forms of metachromatic leukodystrophy. N Engl J Med (1991) 324(1): 18-22.

Qu Y, Miller JB, Desnick RJ, Shapira E. Arylsulfatase A pseudodeficiency: altered kinetic and heat-inactivation properties. Genet Test (1997-98) 1(4): 283-7.

Regis S, Carrozzo R, Filocamo M, Serra G, Mastropaolo C, Gatti R. An AT-deletion causing a frameshift in the arylsulfatase A gene of a late infantile metachromatic leukodystrophy patient. Hum Genet (1995) 96(2): 233-5.

Regis S, Filocamo M, Stroppiano M, Corsolini F, Gatti R. Molecular analysis of the arylsulfatase A gene in late infantile metachromatic leukodystrophy patients and healthy subjects from Italy. J Med Genet (1996) 33(3): 251-252.

Regis S, Filocamo M, Stroppiano M, Corsolini F, Caroli F, Gatti R. A 9-bp deletion (2320del9) on the background of the arylsulfatase A pseudodeficiency allele in a metachromatic leukodystrophy patient and in a patient with nonprogressive neurological symptoms. Hum Genet (1998) 102(1): 50-3.

Regis S, Corsolini F, Stroppiano M, Cusano R, Filocamo M. Contribution of arylsulfatase A mutations located on the same allele to enzyme activity reduction and metachromatic leukodystrophy severity. Hum Genet (2002) 110(4): 351-5.

Resibois A. Electron microscopic studies of metachromatic leucodystrophy. IV. Liver and kidney alterations. Pathol Eur (1971) 6(3): 278-98.

Ricketts MH, Goldman D, Long JC, Manowitz P. Arylsulphatase A pseudodeficiency-associated mutations: population studies and identification of a novel haplotype. Am J Med Genet (1996) 67(4): 387-392.

Ricketts MH, Poretz RD, Manowitz P. The R496H mutation of arylsulfatase A does not cause metachromatic leukodystrophy. Hum Mutat (1998) 12(4): 238-239.

Sandhoff K, Kolter T, Harzer K. Sphingolipid Activator Proteins. In: Scriver CR, et al, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill, 2001:3371-3388.

Sandhoff R, Hepbildikler ST, Jennemann R, Geyer R, Gieselmann V, Proia RL, Wiegandt H, Grone HJ. Kidney sulfatides in mouse models of inherited glycosphingolipid disorders: determination by nano-electrospray ionization tandem mass spectrometry. J Biol Chem (2002) 277(23): 20386-98.

Sawkar AR, Cheng WC, Beutler E, Wong CH, Balch WE, Kelly JW. Chemical chaperones increase the cellular activity of N370S beta -glucosidase: a therapeutic strategy for Gaucher disease. Proc Natl Acad Sci U S A. (2002) 99(24): 15428-33.

Schestag F, Yaghootfam A, Habetha M, Poeppel P, Dietz F, Klein RA, Zlotogora J, Gieselmann V. The functional consequences of mis-sense mutations affecting an intra-molecular salt bridge in arylsulphatase A. Biochem J (2002) 367(Pt 2): 499-504.

Schiffmann R, Murray GJ, Treco D, Daniel P, Sellos-Moura M, Myers M, Quirk JM, Zirzow GC, Borowski M, Loveday K, Anderson T, Gillespie F, Oliver KL, Jeffries NO, Doo E, Liang TJ, Kreps C, Gunter K, Frei K, Crutchfield K, Selden RF, Brady RO. Infusion of alpha-galactosidase A reduces tissue globotriaosylceramide storage in patients with Fabry disease. Proc Natl Acad Sci U S A. (2000) 97(1): 365-70.

Schmidt B, Selmer T, Ingendoh A, Von Figura K. A novel amino acid modification in sulfatases that is defective in multiple sulfatase deficiency. Cell (1995) 82(2): 271-8.

Schott I, Hartmann D, Gieselmann V, Lullmann-Rauch R. Sulfatide storage in visceral organs of arylsulfatase A-deficient mice. Virchows Arch (2001) 439(1): 90-6.

Scott IU, Green WR, Goyal AK, de la Cruz Z, Naidu S, Moser H. New sites of ocular involvement in late-infantile metachromatic leukodystrophy revealed by histopathologic studies. Graefes Arch Clin Exp Ophthalmol (1993) 231(3): 187-91.

Selmer T, Hallmann A, Schmidt B, Sumper M, Von Figura K. The evolutionary conservation of a novel protein modification, the conversion of cysteine to serine semialdehyde in arylsulfatase from *Volvox Carteri*. Eur J Biochem (1996) 238(2): 341-5.

Shapiro LJ, Aleck KA, Kaback MM, Itabashi H, Desnick RJ, Brand N, Stevens RL, Fluharty AL, Kihara H. Metachromatic leukodystrophy without arylsulfatase A deficiency. Pediatr Res (1979) 13(10): 1179-81.

Shen N, Li ZG, Waye JS, Francis G, Chang PL. Complications in the genotypic molecular diagnosis of pseudo arylsulfatase A deficiency. Am J Med Genet (1993) 45(5): 631-7.

Smythe E, Endocytosis in: Subcellular Biochemistry, Volume 27: Biology of the Lysosome, Lloyd and Mason eds., Plenum Press, New York, 1996.

Staellberg-Stenhagen S, Svennerholm L. Fatty Acid Composition Of Human Brain Sphingomyelins: Normal Variation With Age And Changes During Myelin Disorders. J Lipid Res (1965) 79: 146-55.

Stein C, Gieselmann V, Kreysing J, Schmidt B, Pohlmann R, Waheed A, Meyer HE, O'Brien JS, Von Figura K. Cloning and Expression of Human Arylsulfatase A. J Biol Chem (1989) 264(2): 1252-1259.

Suzuki K, Chen GC. Isolation and chemical characterization of metachromatic granules from a brain with metachromatic leukodystrophy. J Neuropathol Exp Neurol (1967) 26(4): 537-50.

Suzuki Y, Toda Y, Tamatani T, Watanabe T, Suzuki T, Nakao T, Murase K, Kiso M, Hasegawa A, Tadano-Aritomi K, et al. Sulfated glycolipids are ligands for a lymphocyte homing receptor, L-selectin (LECAM-1), Binding epitope in sulfated sugar chain. Biochem Biophys Res Commun (1993) 190(2): 426-34.

Suzuki K, Suzuki K. Lysosomal Diseases. In: Graham DI, Lantos PL, eds. Greenfield's Neuropathology. 7th ed. Editor Hodder Arnold, 2002: 653-715.

Svennerholm E, Svennerholm L. Isolation of blood serum glycolipids. Acta Chem Scand (1962) 16: 1282.

Svennerholm L. Some aspects of the biochemical changes in leucodystrophy. In: Folch-Pi J, Bauer H, eds. Brain Lipids and Lipoproteins, and the Leucodystrophies, Elsevier, Amsterdam, 1963: 104.

Takamatsu K. Phytosphingosine-containing neutral glycosphingolipids and sulfatides in the human female genital tract: their association in the cervical epithelium and the uterine endometrium and their dissociation in the mucosa of fallopian tube with the menstrual cycle. Keio J Med (1992) 41(3): 161-7.

Takashima S, Matsui A, Fujii Y, Nakamura H. Clinicopathological differences between juvenile and late infantile metachromatic leukodystrophy. Brain Dev (1981) 3(4): 365-74.

Tesluk H, Munn RJ, Schwartz MZ, Ruebner BH. Papillomatous transformation of the gallbladder in metachromatic leukodystrophy. Pediatr Pathol. (1989) 9(6): 741-6.

Toda K, Kobayashi T, Goto I, Kurokawa T, Ogomori K. Accumulation of lysosulfatide (sulfogalactosylsphingosine) in tissues of a boy with metachromatic leukodystrophy. Biochem Biophys Res Commun (1989) 159(2): 605-11.

Toda K, Kobayashi T, Goto I, Ohno K, Eto Y, Inui K, Okada S. Lysosulfatide (sulfogalactosylsphingosine) accumulation in tissues from patients with metachromatic leukodystrophy. J Neurochem (1990) 55(5): 1585-91.

Tominaga L, Ogawa Y, Taniguchi M, Ohno K, Matsuda J, Oshima A, Suzuki Y, Nanba E. Galactonojirimycin derivatives restore mutant human beta-galactosidase activities expressed in fibroblasts from enzyme-deficient knockout mouse. Brain Dev (2001) 23(5): 284-7.

Tsuda T, Hasegawa Y, Eto Y. Two novel mutations in a Japanese patient with the late-infantile form of metachromatic leukodystrophy. Brain Dev (1996) 18(5): 400-3.

Van den Hout JM, Reuser AJ, de Klerk JB, Arts WF, Smeitink JA, Van der Ploeg AT. Enzyme therapy for pompe disease with recombinant human alpha-glucosidase from rabbit milk. J Inherit Metab Dis. (2001) 24(2): 266-74.

Von Bulow R, Schmidt B, Dierks T, von Figura K, Uson I. Crystal structure of an enzyme-substrate complex provides insight into the interaction between human arylsulfatase A and its substrates during catalysis. J Mol Biol (2001) 305(2): 269-77.

Von Bulow R, Schmidt B, Dierks T, Schwabauer N, Schilling K, Weber E, Uson I, von Figura K. Defective oligomerization of arylsulfatase a as a cause of its instability in lysosomes and metachromatic leukodystrophy. J Biol Chem (2002) 277(11): 9455-61.

Von Figura K, Steckel F, Hasilik A. Juvenile and adult metachromatic leukodystrophy: Partial restauration of arylsulfatase A (cerebroside sulfatase) activity by inhibitors of thiol proteinases. Proc Natl Acad Sci (1983) 80:6066-6070.

Von Figura K, Hasilik A. Lysosomal enzymes and their receptors. Ann Rev Biochem (1986) 55: 167-193.

Von Figura K, Schmidt B, Selmer T, Dierks T. A novel protein modification generating an aldehyde group in sulfatases: its role in catalysis and disease. Bioessays (1998) 20(6): 505-10. Review.

Von Figura K, Gieselmann V, Jaeken J. Metachromatic leukodystrophy. In: Scriver CR, et al, eds. The metabolic and molecular basis of inherited disease. 8th ed. New York: McGraw-Hill, 2001:3695-3724.

Waheed A, Hasilik A, von Figura K. Synthesis and processing of arylsulfatase A in human skin fibroblasts. Hoppe Seylers Z Physiol Chem (1982) 363(4): 425-30.

Waldow A, Schmidt B, Dierks T, von Bulow R, von Figura K. Amino acid residues forming the active site of arylsulfatase A. Role in catalytic activity and substrate binding. J Biol Chem (1999) 274(18): 12284-8.

Wolfe Hj, Pietra Gg. The Visceral Lesions Of Metachromatic Leukodystrophy. Am J Pathol (1964) 44: 921-30.

Zhu XH, Hara A, Taketomi T. The existence of galactosylceramide I3-sulfate in serums of various mammals and its anticoagulant activity. J Biochem (1991) 110(2): 241-5.

Zlotogora J, Bach G, Barak Y, Elian E. Metachromatic leukodystrophy in the habbanite Jews: high frequency in a genetic isolate and screening for heterozygotes. Am J Hum Genet (1980) 32(5): 663-9.

Zlotogora J, Furman-Shaharabani Y, Goldenfum S, Winchester B, von Figura K, Gieselmann V. Arylsulfatase A pseudodeficiency: A common polymorphism wich is associated with a unique haplotype. Am J Med Genet (1994a) 52: 146-150.

Zlotogora J, Furman-Shaharabani Y, Harris A, Barth ML, von Figura K, Gieselmann V. A single origin for the most frequent mutation causing late infantile metachromatic leucodystrophy. J Med Genet (1994b) 31: 672-674.

Zlotogora J, Bach G, Bosenberg C, Barak Y, von Figura K, Gieselmann V. Molecular basis of late infantile metachromatic leukodystrophy in the Habbanite Jews. Hum Mutat (1995) 5(2):137-43.