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**SET UP AND OPTIMIZATION OF A TANDEM MASS SPECTROMETRY
PROCEDURE FOR ENZYMATIC ASSAYS OF LYSOSOMAL STORAGE
DISORDERS AND FEASIBILITY EVALUATIONS FOR A NEWBORN
SCREENING PROGRAM**

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SUMMARY

LSDs are a cluster of about 50 different inheritable pathologies mainly due to the deficit of lysosomal enzymes, which lead to severe and progressive multiorgan dysfunction. Biochemically well defined, they are characterized by an ubiquitous accumulation of undegraded macromolecules and, although individually rare, they present an overall incidence around 1 in 1500-7000 newborns.

Main therapies for these pathologies are Enzyme Replacement and Hematopoietic Stem Cell Transplantation, both needing an early application to maximize their benefits; up to now, laboratory tests are carried out only following a particular clinical suspect and they allow a correct diagnosis when symptoms (sometimes irreversible) are already clearly developed.

Other medical approaches, as enzyme enhancement therapy, substrate deprivation/reduction therapy and gene therapy, are on trial.

Moreover, still very little is known on the individual determinants of therapeutic efficacy and, at the moment, the same therapeutic scheme is applied to all patients, although it is becoming clear that much better results could be obtained for each single patient if personalized therapeutic protocols could be defined.

Both of these questions drive to the search of new diagnostic approaches able to detect patients in the population at the earliest possible time, in order to allow a very precocious application of therapies, and able to rapidly test enzymes bio-availability in blood, to facilitate the development of personalized therapeutic protocols, to reduce unnecessary treatments and side effects and to lead to a more cost-effective healthcare.

Finally, recent studies on high number of samples, have reported an unexpected high incidence of these disorders, underlined how LSDs might have been since now underestimated.

For all these reasons in the last years a new easy, cheap, and reliable diagnostic test, using Tandem Mass Spectrometry, was developed allowing to measure enzymatic activities in Dried Blood Spots (DBS).

This work was addressed to the set up and optimization of this method for the detection of the enzymatic activities for six lysosomal storage disorders; the

choice of the diseases to take into consideration in this study was made according to the availability of a therapy and of reagents for MS/MS analysis.

The first part of the work was conducted at the Centogene Laboratory of Vienna; after the Gaucher and Niemann Pick A/B assays set up, they were multiplexed in a single test together with Pompe, Fabry and Krabbe assays. Then, the reliability and sensitivity of the method were investigated through a cross comparison work between two laboratories (the Vienna Centogene Laboratory and a research group of the Wadsworth Center of NY). An evaluation of the most critical steps of the protocol that needed to be optimized was made.

Finally, a pilot newborn screening project was started in collaboration with the Hospital of Szeged. Ten thousand Hungarian anonymous samples were analyzed singly, then samples presenting low activities for one of the enzymes were re-tested in duplicate. Confirmed positive samples were sent to a third laboratory in Rostock for molecular analysis, so the detection of mutation for pathological samples and an evaluation of the false positive rate was possible.

These studies confirm, not only the reliability of using DBS as biological specimens and the feasibility of the analysis by tandem mass spectrometry, but also the easy application of the method for screening intent.

The second part of the work was carry out in Padova, in collaboration with the Tandem Mass Spectrometry Laboratory of the Department of Pediatrics.

Here the multiplex assay was reproduced and the test for Mucopolysaccharidosis type I was added.

Considering the preliminary results obtained in Vienna the protocol has been modified, by reducing the sample manipulation, through the introduction of an Ultra Performance Liquid Chromatography connected in line with the MS/MS spectrometer, and by optimizing the analytical parameters.

The aim of the work was to maximize MS/MS sensitivity, precision and accuracy, to allow all enzyme activities to show an unambiguous difference between DBS obtained from healthy controls and LSD affected patients.

The new defined protocol for enzyme activity detection described in this thesis will be potentially applied for several different purpose: it can be used both for the screening of wide populations, with the possibility to add new enzymatic tests for

those disorders that will have a therapy available, or for the enzyme bioavailability determination in single patients.

RIASSUNTO

Le malattie da accumulo lisosomiale (LSD) rappresentano un gruppo di circa 50 disordini genetici, dovuti principalmente al deficit di specifiche idrolasi con conseguente accumulo di substrati non degradati nei lisosomi di organi e tessuti. Le LSD sono malattie molto complesse, gravemente debilitanti o letali, caratterizzate da un'estrema variabilità per quanto riguarda età di esordio, sintomatologia e rapidità di decorso clinico.

Pur essendo singolarmente rare, esse presentano un'incidenza complessiva stimata di 1:1500-7000 nati vivi.

Le principali terapie a disposizione, oltre alle cure palliative, sono la terapia enzimatica sostitutiva (ERT) e il trapianto di cellule staminali ematopoietiche; entrambe presentano massima efficacia se iniziate precocemente, prima cioè dell'insorgenza delle principali manifestazioni cliniche. Tuttavia, spesso una diagnosi precoce non è possibile poiché i test di laboratorio vengono eseguiti solo in seguito ad un evidente sospetto clinico. Altre strategie terapeutiche in fase di trial comprendono l'enzyme enhancement therapy, la terapia da inibizione/riduzione del substrato e la terapia genica.

Ad oggi inoltre, i soggetti in trattamento vengono tutti sottoposti allo stesso protocollo terapeutico; sarebbe invece opportuno riuscire a sviluppare protocolli terapeutici personalizzati andando a valutare rapidamente la biodisponibilità dell'enzima somministrato, durante il follow up dei pazienti.

Entrambe queste problematiche hanno portato, negli ultimi anni, alla ricerca di nuove tecniche diagnostiche, sia adatte per l'analisi di un elevato numero di campioni, che rispondenti alla necessità di permettere una veloce stima dell'attività degli enzimi di interesse in singoli soggetti.

Il dosaggio enzimatico su Dried Blood Spot (DBS) mediante spettrometria in tandem massa si è rivelato un metodo ideale per la diagnosi delle malattie lisosomiali, poiché esso si distingue per affidabilità, sensibilità, rapidità e accuratezza e consente l'analisi enzimatica multipla in un elevato numero di campioni contemporaneamente.

Infine, studi recenti di screening per alcune LSD condotti sia su ampie popolazioni sia su specifici gruppi a rischio (pazienti con infarto criptogenico o

con insufficienza renale) hanno riportato un'incidenza complessiva sorprendentemente elevata, oltre a diversi casi, in particolare della malattia di Fabry, di mancato riconoscimento della patologia; ciò prova come questi disordini siano stati fino ad oggi sottovalutati, e sottolinea l'importanza di una corretta e tempestiva diagnosi.

Questo lavoro è dedicato alla messa a punto e all'ottimizzazione di questa nuova metodica per lo studio dell'attività enzimatica per sei malattie da accumulo lisosomiale (Pompe, Fabry, Niemann Pick A/B, Gaucher, Krabbe e Mucopolisaccaridosi di tipo I). La scelta delle patologie è stata determinata sia, per ragioni etiche, dalla disponibilità di una terapia possibile, sia dalla disponibilità dei reagenti per la MSMS da parte del CDC di Atlanta.

La prima parte del lavoro è stata svolta presso il laboratorio Centogene di Vienna, e ha previsto la messa a punto dei saggi per la malattia di Gaucher e Niemann Pick A/B, che sono stati successivamente unificati in un unico test insieme ai saggi per le malattie di Pompe, Fabry e Krabbe.

In seguito, uno studio incrociato tra due laboratori (il laboratorio Centogene di Vienna e un gruppo di ricerca del Wadsworth Center di NY), su più di 400 campioni ha permesso di valutare l'affidabilità e la sensibilità della tecnica utilizzata, oltre ad individuare i passaggi critici del protocollo che sarebbero stati in seguito ottimizzati.

Infine, nel corso di questo progetto è stato iniziato uno studio pilota di screening neonatale in collaborazione con l'Ospedale di Szeged (Ungheria). Diecimila campioni anonimi sono stati analizzati singolarmente; i campioni che presentavano una bassa attività per uno degli enzimi di interesse sono stati ri-analizzati in duplicato e nel caso di conferma, inviati ad un terzo laboratorio (Centogene di Rostock) per l'analisi molecolare. In questo modo è stata possibile sia un'identificazione delle mutazioni nei campioni patologici, sia una valutazione della percentuale di falsi positivi.

Questi lavori hanno confermato non solo l'affidabilità nell'utilizzo dei DBS come campione biologico e la sensibilità della spettrometria in tandem massa per l'analisi, ma anche la facile organizzazione e applicazione di tale metodica per progetti di screening.

La seconda parte del progetto è stata svolta a Padova in collaborazione con la sezione di spettrometria di massa del Dipartimento di Pediatria.

Qui è stato riprodotto il saggio multiplo per i 5 enzimi sopra citati ed è stato aggiunto inoltre il test per la Mucopolisaccaridosi di tipo I.

Tenendo conto dei risultati preliminari ottenuti a Vienna il protocollo è stato così modificato: è stata ridotta al minimo la parte preparativa dei campioni, sostituita da una cromatografia liquida collegata online con lo spettrometro di massa; inoltre, tutti i parametri analitici sono stati minuziosamente ottimizzati. Lo scopo è stato quello di massimizzare la sensibilità e precisione di rilevamento degli analiti per garantire una inconfutabile discriminazione tra soggetti sani e patologici.

Questa nuova metodica può essere applicata per scopi diversi: sia per lo screening di ampie popolazioni, con la possibilità di aggiungere nuovi test al pannello delle malattie screenate via via che sarà per esse disponibile una terapia, sia per una rapida valutazione della bio-disponibilità dell'enzima in singoli soggetti.

1.INTRODUCTION

1.1 The Lysosome

The lysosome received its name in 1955 thanks to the work of the doctor and scientist Christian De Duve and his co-workers. Lysosome was first described as a “sac-like particle surrounded by a membrane that prevented the enzyme from getting out and the substrate from getting in” [1]; moreover, the discovery of five acid hydrolases, acting on different substrates, suggested the digestive function of the structure.

In 1963 J. Danielli (the father of the bi-layer concept) supervised the first international symposium on lysosomes, where the terms “endocytosis”, “autophagy” and “exocytosis” have been coined [2]. After this milestone, the studies on that field expanded very rapidly and led in 1969 to the publication of a multi-author work “Lysosome in biology and pathology” [3].

The very first lysosomal deficit was hypothesized by Hers, in 1961: it involved the enzyme α -Glucosidase, responsible of the hydrolization of glycogen; Hers first proposed the concept of "inborn lysosomal disease", which he defined as a condition in which the genetic deficiency of a lysosomal enzyme leads to the intra-lysosomal accumulation of all the materials that are normally degraded by this enzyme [4].

Nowadays, the structures, the functions and the activity mechanisms of lysosomal enzymes are commonly known. Synthesized in the Golgi apparatus, they are modified with mannose-6-phosphate (M6P) that, being recognized by specific M6P receptors allows them to be transferred via endosome to lysosomes, where they accomplish their catalytic function.

Since there are many steps necessary for synthesis and processing of lysosomal enzymes, errors can result at many different steps in the pathway.

1.2 Lysosomal Storage Disorders

Lysosomal storage disorders (LSDs) are identified as a group of more than fifty rare genetic diseases with a combine incidence of 1:1500-7000 live births [5].

The deficit of one or more lysosomal enzyme activities or, in few cases, of non-enzymatic lysosomal proteins, or non-lysosomal proteins involved in lysosomal

biogenesis, lead to the storage of undegraded substrates in lysosomes of different cell types.

LSDs present an autosomal recessive inheritance, except for the X-linked Fabry Disease Mucopolysaccharidosis type II and Danon Disease. Although normally monogenic, for most LSDs many mutations have been described in the same gene, leading to a really wide spectrum of symptoms, age of onset (but mainly the pediatric age) and degree of severity, preventing any possibility of genotype-phenotype correlation [6,7].

1.2.1 LSDs classification

The LSDs most common classification has been proposed considering the type of accumulated material in cells; moreover the age of onset and disease severity are discriminating for subgroups division.

Defective degradation of glycogen:

- Infantile-onset Pompe Disease (α -Glucosidase deficiency)
- Late-onset Pompe Disease

Defective metabolism of glycosaminoglycans (Mucopolysaccharidosis)

- MPS type IH, Hurler syndrome (α -L-Iduronidase deficiency)
- MPS type I H/S, Hurler-Scheie Syndrome
- MPS type IS, Scheie Syndrome
- MPS type II A, Hunter Syndrome, severe (Iduronate Sulfatase deficiency)
- MPS type II B, Hunter Syndrome, mild (Iduronate Sulfatase deficiency)
- MPS type III A-D, Sanfilippo Syndrome (Heparan *N*-Sulfatase deficiency)
- MPS type IV A, Morquio Syndrome, classic (Galactose 6-Sulfatase deficiency)
- MPS type VI, Maroteaux-Lamy Syndrome (Arylsulfatase B deficiency)
- MPS type VII, Sly Syndrome (β -Glucuronidase deficiency)

Defective degradation of glycan portion of glycoproteins

- α -Mannosidosis and β -Mannosidosis (α -Mannosidase and β -Mannosidase deficiency)
- α -Fucosidosis I and II (α -Fucosidase deficiency)
- Sialidosis I and II (α -*N*-Acetyl Neuraminidase deficiency)
- Aspartylglucosaminuria (Aspartylglucosaminase deficiency)

Defective degradation of sphingolipid components

- Niemann Pick Disease type A and B (Acid-Sphingomyelinase deficiency)

- Fabry Disease (α -Galactosidase deficiency)
- Farber Disease (acid-Ceramidase deficiency)
- Gaucher Disease I, II, III (β -Glucosidase deficiency)
- GM1 Gangliosidose I, II, III (β -Galactosidase A deficiency)
- Tay-Sachs Disease I, II, III (β -hexosaminidase (α chain) deficiency)
- Sandhoff Disease (β -Hexosaminidase (β chain) deficiency)
- Krabbe Disease (Galactocerebrosidase deficiency)
- Metachromatic Leukodystrophy I, II (Arylsulfatase A (ASA) deficiency)

Defective degradation of polypeptides

- Pycnodystosis (cathepsin k deficiency)

Defective degradation or transport of cholesterol, cholesterol esters, or other complex lipids

- Neuronal Ceroid Lipofuscinosis I, (palmitoyl-protein thioesterase deficiency)
- Neuronal Ceroid Lipofuscinosis II (deficiency of the acid protease tripeptidyl-peptidase (TPP-1))
- Neuronal Ceroid Lipofuscinosis III (LN3 protein ("Battenin"))
- Neuronal Ceroid Lipofuscinosis IV (deficiency of palmitoyl-protein thioesterase 1 (PPT1))
- Niemann Pick C (NPC1/2 Protein deficiency)

Multiple deficiencies of lysosomal enzymes

- Galactosialidosis (32-kilodalton protein deficiency)
- Mucopolipidosis II, III (UDP-N-acetylglucosamine-1-phosphotransferase deficiency)

Transport and trafficking defects

- Cystinosis (Cystinosin deficiency)
- Mucopolipidosis IV (Unknown, but likely ganglioside sialidase (neuraminidase) deficiency)
- Infantile Sialic acid storage disease (ISSD) (sialic acid transporter deficiency)
- Salla Disease (sialin protein deficiency)

1.2.2 Therapies and diagnostic tests

Lysosomal storage disorders may manifest at every age. The infantile forms, most severe, present acute brain involvement and patients generally die within the first years of life.

Instead in adult forms symptoms develop more slowly and with less severe disabilities. Intermediate juvenile forms are also common.

Neurological symptoms can include seizures, dementia and brainstem dysfunction. Main peripheral symptoms consist in hepatosplenomegaly, heart and kidney injury, abnormal bone formation, muscle atrophy and ocular disease.

However each LSD has a quite distinct clinical and pathological picture which partly relates to the nature of the accumulated material and to the cell type in which it accumulates [8].

In some diseases different levels of enzyme activity may be the cause of phenotypic variability, but in other cases there is no obvious genotype-phenotype correlation, rather patients that present the same genetic background may show widely different clinical symptoms or even be asymptomatic for the disease [9].

The overlap of LSD's symptoms to those of other most common diseases often makes the diagnosis very long (months or even years) and complex, limiting the benefits of available therapies. Generally after a particular clinical suspect several laboratory tests could be carried out to get the right diagnosis.

Urine tests are performed for quantitative and qualitative analysis of glycosaminoglycans (GAGs) and oligosaccharides in patients suspected to have an MPS, or a disorder that presents with oligosaccharinuria, such as I-cell Disease, Mucopolipidosis type 3, GM1 and GM2 Gangliosidosis, Fucosidosis, α -Mannosidosis, Sialidosis, Galactosialidosis and infantile Sialic Acid Storage.

Although urine tests are very sensitive, affected individuals with normal urine have been reported.

Other diagnostic exams entail enzyme analysis of either leukocytes or cultured skin fibroblasts; they predominantly include enzymes involved in the digestion of glycosphingolipids and oligosaccharides (Gaucher Disease, Niemann Pick Type A and B, Pompe Disease, MPSs, Acid Lipase Deficiency, GM1 and GM2, Krabbe Disease, Metachromatic leukodystrophy, Mucopolipidosis type 2 and 3, Fucosidosis, α -Mannosidosis and Schlinler Disease) [10].

In the cases in which defects in secondary or activator proteins occur, the diagnosis can be achieved by determination of radiolabeled substrate turnover in cultured cells; for example in Niemann Pick type C Disease it is possible to analyze cholesterol processing and accumulation in cultured fibroblasts [11].

For all these disorders molecular analysis provide the confirmatory test and, alone or combined with the determination of enzyme activity in fetal amniotic fluid cells, it allows a rapid, sensitive and accurate prenatal diagnosis for some LSDs, as it has been shown in several studies [12,13,14,15].

All these diagnostic methods could be very useful for specific suspected patients, but they are not feasible for a great number of samples as in the case of specific high risk population or for newborn screening intent.

On the contrary the need to perform screening tests at least for some of these pathologies is becoming mandatory, considering that the effectiveness of therapies, particularly for those LSDs involving the Central Nervous System (CNS) and bone pathologies, will strictly rely upon an early treatment, before the onset of irreversible damages.

Recombinant functional enzymes have been developed for the so called Enzyme Replacement Therapy (ERT), based on the premise that only 1% to 5% of enzyme activity is sufficient to correct many of these defects and on the capability of somatic cells to internalize exogenous enzymes (via the mannose-6 receptor pathway or via the macrophage mannose receptors) and to transfer them to the lysosome where they degrade accumulated substrate [16,8].

ERT is at the moment available for Gaucher Disease type 1, Fabry Disease, MPS type I, II and VI, Pompe Disease while clinical trials for Niemann Pick A/B and MPS IVA are in progress. The big limitation of this therapy, in addition to the elevated costs, is its incapacity to correct central nervous system (CNS) manifestations given the inability of the enzymes to cross the blood brain barrier. Moreover, even if important results have been obtained for the majority of patients, the extent of response seems to vary a lot from individual to individual.

Other medical approaches mainly include deprivation/reduction therapy (SDT/SRT) [17], enzyme enhancement therapy (EET) [18], gene therapy [19] and haematopoietic stem cell transplantation (HSCT) [20].

SRT aims to reduce substrate accumulation by partially inhibit its biosynthetic circle. Between the inhibitors, Miglustat (N-butyl-deoxynojirimycin) has been approved for use in patients with Gaucher type 1, but it is recommended only for adults in combination with ERT or when ERT is not a possibility; under experiment patients with Gaucher type3, Niemann Pick type C, GM2 gangliosidosis [21].

In some specific mutants, yet catalytically active, enzymes EET is used and involves small molecule chaperones that reversibly bind the active site of the enzyme, correcting the misfolding and allowing the delivery to lysosomes. This strategy is being tested for Fabry, Gaucher type1, Pompe, GM1 gangliosidosis, Tay-Sachs and Sandhoff Diseases [22].

HSCT or bone marrow transplantation is also a valid therapeutic option, which success strongly depends on the precocity of intervention, and on the enzyme deficiency. It has been successfully used in MPS I, VI, VII and Krabbe Disease.

Another potential treatment under development is gene therapy, that may have the advantage to provide the functional enzyme for long time in all involved organ systems. Studies with both viral or non-viral vectors have been performed in animal models but these protocols are not ready yet for clinical trials in patients.

In general, all therapeutic strategies would need an early application to maximize their benefits [23,24]. Moreover, an early and accurate diagnosis is imperative for genetic counselling of the parents and may provide them with reproductive choice; finally, but not less relevant, a rapid test would avoid the prolonged, stressful diagnostic “odyssey”.

At the beginning of the last decade the work of Chamoles and co-workers led to the introduction of Dried Blood Spots (DBS) for enzymatic diagnosis of several LSDs, demonstrating that activities of many lysosomal enzymes remain stable after elution from DBS and can be measured using artificial fluorescent tag substrates (e.g. 4-methylumbelliferone) [25] linked to an analogue of the natural substrate, that does not fluoresce unless cleaved.

DBS, introduced for different purposes by the pioneering work of Dr. Robert Guthrie in the '60s, have represented the real innovation for the diagnosis of LSDs since, being simple blood drops spotted onto specific filter paper with little invasiveness, they allow an easy delivery of the samples at room temperature, with no problems of deterioration, thus favouring the creation of a few centralized reference laboratories for their analysis. Nowadays DBS are used to screen newborns for more than 50 treatable inborn diseases [26].

In particular, with respect to LSDs, this method allowed so far to set up reliable tests for many pathologies as Pompe, Fabry, Gaucher, Niemann Pick A and B Diseases, GM1 gangliosidosis, Tay-Sachs and Sandhoff Diseases and some MPSs, but the main advance in this direction has been provided by the

application of the Tandem Mass Spectrometry, which renders the analysis more robust and even faster, performing multiple assays for the contemporary analysis of several pathologies, in an elevated number of samples [27].

1.2.3 Newborn screening pilot studies

Several newborn screening pilot studies have been carried out.

In Austria Dajnoki and co-workers set up the MS/MS method for high-throughput analysis and newborn screening of Pompe Disease in more than 10000 anonymous newborns [28]; moreover, in 2005, a screening program for Pompe Disease was conducted in Taiwan (where this pathology is more frequent than in the world-wide population) on 344056 newborns [29]. Four patients, who were less than one month of age, were identified and could immediately initiate the therapy; thus resulting in normal cardiac functions, growth and acquisition of age appropriate milestones [30].

Also in Taiwan, a pilot project for Fabry reported an incidence of 1~1250 males of which 86% carried mutations associated with a late onset phenotype [31].

In 2006 New York was the first state that approved the screening for Krabbe disorders to allow early diagnosis of the infantile form of the disease to be followed by HSCT. In 2009, after three years of screening, they defined cutoffs, a testing algorithm and a long term follow up study for moderate and high risk children [32]. Moreover, the National Institutes of Health and Genzyme Corporation is supporting in Washington State a pilot study to detect Pompe, Fabry, and MPS I disorders.

In Italy, in 2004 LSDs were included in the screening program of the Tuscany Region, with so promising results that in 2006 also part of the Umbria region joined the project [33].

In the last few years the challenge was to assess the feasibility of a multiplex assay for at least six disorders [27] with very promising results [34,35].

Very recently Thomas Mechtler and co-workers reported the outcome of an anonymised neonatal screening in Austria for a group of four LSDs (Pompe, Fabry, Niemann Pick A/B and Gaucher Disease). 34736 blood spots from neonates were tested in 2010 in a 7 months period; positive samples went through molecular analysis. The study revealed a higher than expected birth

incidence (on average 1:2315), with a high frequency of mutations associated with late onset forms [36].

Being LSDs monogenic and globally well characterized disorders, for some of which the availability of animal models can help the preclinical evaluation for pathophysiology studies, they could be defined an ideal model of Rare Disorder, to be included in newborn screening programs. In addition, the recent rapid development of therapeutic strategies for this group of pathologies would be meaningless without the possibility of a precocious application, which could be importantly guaranteed by a newborn screening program.

Under the pressure of the rapid spread of LSD screening programs in several countries, The Newborn Screening Quality Assurance Program (NSQAP) at the US Center for Disease Control and Prevention (CDC, <http://www.cdc.gov/labstandards/nsqap.htm>) has already distributed quality controls and standards to more than 70 laboratories, playing a pivotal role in maintaining and enhancing the quality of LSD newborn screening test results.

A preliminary Quality assurance (QA) study involving 16 laboratories indicates a coefficient of variation between different units of 20% [37].

The importance of newborn screening programs has been previously demonstrated in several works conducted on specific ethnic groups characterized by high consanguinity (especially on Jewish and Turkish populations).

In Israel, the Dor Yeshorim organization [38] offers screening for genetic diseases for people belonging to the Ashkenazi Jewish population: a significant impact, in particular by reducing the number of cases of Tay–Sachs Disease, was reported [39]. The same organization carries out anonymous genetic screening of couples before marriage in order to reduce the risk of children affected by genetic diseases [40].

1.3 Diseases included in the study

1.3.1 Pompe Disease

It was first described by Dr. J.C. Pompe in 1932. Pompe Disease (OMIM 232300) is an autosomal recessive disorder with an incidence of approximately 1:40000 live births. Mutations in the gene α -glucosidase (GAA) (EC: 3.2.1.20) result in reduced or absent enzyme activity (Hydrolysis of terminal, non-reducing (1->4)-

linked α -D-glucose residues with release of α -D-glucose) and leads to glycogen accumulation in different cell types, mainly in skeletal muscle and heart.

Pompe Disease presents different phenotypes that swept from an infantile, most severe form to a slower late-onset form [41]. The first one is characterized by hypertrophic cardiomyopathy and a generalize muscle weakness often followed by cardio respiratory failure or respiratory infection that lead to death within the first year of life; on the contrary the late onset forms present no cardiac symptoms and the skeletal muscle weakness is slowly progressive [42].

Molecular aspects

The α -Glucosidase gene is located on chromosome 17 (17q25.2-q25.3), and it is constituted by 19 coding exons in 20kB of genomic DNA producing a 952 aa protein.

There are three common alleles of GAA: GAA*1 (the most common), GAA*2 and GAA*4 (<http://www.uniprot.org/uniprot/P10253>).

More than 200 mutations have been described [43].

(<http://cluster15.erasmusmc.nl/klgn/pompe/mutations.html>)

Treatment

The first treatment for Pompe patients was Myozyme, that in March 2006 received marketing authorization in the European Union and one month later the FDA approval in the Unites States. Myozyme is a recombinant human acid α -glucosidase derived from Chinese Hamster Ovary cells (CHO-cells) and is used in a protocol of enzyme replacement therapy (ERT) [44].

Other therapeutic approaches are under investigation: Enzyme Enhancement Therapy (EET) and Gene Therapy. The former aims to restore α -Glucosidase activity in the lysosomes of patients that produce a misfolded protein. The effect of chemical chaperones has so far only been tested in cultured fibroblasts from patients with Pompe Disease [45].

Gene therapy with adenoviral (Ad), adeno-associated (AAV) and hybrid Ad-AAV vectors has been developed in rat, mice and quail. The results in animal models are promising, but still several important aspects, regarding immune response control, sustained expression of the transgene or safety have to be investigated [46].

1.3.2 Fabry Disease

Fabry Disease (also called Anderson-Fabry Disease, OMIM 301500) was first described in 1898 by two dermatologists working independently: Dr. Dr William Anderson in England and Dr Johannes Fabry in Germany. It is an X-linked disorder with an incidence from 1 to 5 in 100000; according to several studies, on specific populations (kidney transplant patients, patients with early-onset cryptogenic stroke or with hypertrophic cardiomyopathy) true prevalence may be underestimated, as they identified previously unrecognized individuals with Fabry Disease [47].

The deficiency of the α -Galactosidase A (GLA) (E.C. 3.2.1.22) activity (hydrolysis of terminal, non-reducing α -D-galactose residues in α -D-galactosides, including galactose oligosaccharides, galactomannans and galactolipids) leads to a progressive multisystemic disorder due to the accumulation of globotriaoslyceramide (Gb3) and related glycosphingolipids in the plasma and cellular lysosomes throughout the body.

Symptoms typically arise in childhood between the age of four and ten years and are characterized by acute and chronic pain, tingling and paresthesias of the extremities; with increasing age very frequent are cardiac dysfunctions, renal failure and cerebrovascular abnormalities. Heterozygous females can present a wide range of symptom's severity, from very mild to severe. Late-onset presentations with residual GLA activity and predominantly cardiac or renal manifestations have also been described [48].

Molecular aspects

The α -Galactosidase A gene is located on the X chromosome (Xq22), it has a length of 10.222 nucleotides and consists of seven exons and six introns producing a 429 aa protein.

492 mutations in this gene, associated with Fabry Disease, are known (HGMD), moreover one mutation is not clearly associated with Fabry and one mutation leads to an increased transcription of GLA. About 70% are missense or nonsense mutations [49].

(<http://www.uniprot.org/uniprot/P06280>)

Treatment

ERT is available since 2001; Fabrazyme is produced by Genzyme by recombinant DNA technology in a Chinese Hamster Ovary mammalian cell expression system [50].

Enzyme enhancement therapy is on trial with galactose [51]

1.3.3 Krabbe Disease

The disease (OMIM 245200) is named for the Danish neurologist Knud Haraldsen Krabbe, who described it in 1913-16. It is an autosomal recessive disorder with a global incidence of 1: 100000 live birth [52].

Krabbe Disease is caused by the deficit of the enzyme Galactocerebrosidase (GALC, EC=3.2.1.46) responsible of the hydrolyzation of the galactose ester bonds of galactosylceramide, galactosylsphingosine, lactosylceramide, and monogalactosyldiglyceride, that are important in the production of normal myelin; demyelination causes physical disabilities, while mental abilities are not hindered. The disease could manifest very early in life (three-six months) with sudden onset irritability, startle response to stimuli and developmental arrest; most of affected infants do not survive beyond two years of age. Late-onset forms could develop in childhood or in adulthood with similar symptoms and a rapid unlucky onset [53].

Molecular aspects

GALC gene is located on chromosome 14 (14q31) and consists of 17 exons spanning about 60 kb of genomic DNA, encoding for a 685 aa protein [54].

More than seventy pathogenic mutations are known, the most frequent of which is a 30kb deletion starting in intron 10 and extending beyond the end of the gene, associated with an early onset form of the disease.

(<http://www.uniprot.org/uniprot/P54803>)

Treatment

Treatment for Krabbe Disease is currently limited to pre-symptomatic hematopoietic stem cell transplantation (HSCT) typically using umbilical cord blood, unfortunately a procedure with a 10% mortality and significant morbidity [24].

1.3.4 Gaucher Disease

The disease (OMIM 230800, 230900, 231000) is due to the French doctor Philippe Gaucher, who originally described it in 1882. It is an autosomal recessive disorder caused by the deficit of the activity of the enzyme acid- β -Glucosidase (ABG; E.C. 3.2.1.45), responsible for the degradation of glucocerebroside into glucose and ceramide. Undegraded molecules lead to accumulation and storage of glucosylceramide in cells of mononuclear origin. Gaucher Disease (GD) is the most common disorder among LSDs, with a global incidence of about 1:57000 but may be as frequent as 1:855 in the Ashkenazi Jewish population [55].

Gaucher Disease comprises three subtypes: type 1 is the non-neuropathic form, it could manifest early in life as well as in adulthood and it includes hepatosplenomegaly, skeletal weakness and bone disease; many patients have a mild form of the disease or may not show any symptoms. Type 2 is the acute infantile neuropathic form, and it is characterized by hepatosplenomegaly, extensive and progressive brain damage, eye movement disorders, spasticity, seizures, limb rigidity; affected children usually die by the age of 2. Finally, type 3 can show up at any time in childhood or even in adulthood and presents slowly progressive but milder neurologic symptoms, enlarged spleen and/or liver, seizures, poor coordination, skeletal irregularities, eye movement disorders, blood disorders, including anemia, and respiratory problems; patients often survive up to their early teen years or adulthood [56,57].

Molecular aspects

ABG gene is constituted by 11 exons located on Chromosome 1 (1q22), encoding for a 536 aa protein.

There are nearly 200 known mutations, mostly missense, which result in substitution of amino acids in the protein. Some mutations cause complete deactivation of the enzyme; others impair its stability, and some affect both activity and stability.

(<http://www.uniprot.org/uniprot/P04062>).

Treatment

Recombinant enzyme for ERT received FDA approval in 1994, and it is produced by Genzyme (Cerezyme™) in CHO cells or in a carrot stem cells suspension [58]. Recently an FDA-approved Early Access Program (EAP) allowed the use of a new drug, pre-licensed ERT, called velaglucerase alfa (VPRIV™, Shire HGT, Cambridge MA, USA) [59].

Bone marrow or hematopoietic stem-cell transplantation are also applied, but limited because of shortage of adequate donors and procedural risks; moreover HSCT results really effective if performed before the development of irreversible skeletal and organ changes [60].

Other therapeutic approaches in trial are substrate reduction therapy and gene therapy. For the first strategy a small molecule inhibitor (Zavesca™) is used to inhibit the synthesis of the accumulated glucosylceramide: it is indicated for the treatment of adult patients with mild to moderate type 1 Gaucher Disease for whom enzyme replacement therapy is not a therapeutic option, due to immunological response [61].

Retroviral gene transfer has been tested with success in GD1 mice (Enquist et al, anno) and could be used to correct the GBA deficit in primary hematopoietic cells from patients with Gaucher Disease [62].

1.3.5 Niemann Pick A/B Disease

Albert Niemann published the first description of what is now known as Niemann–Pick Disease, type A, in 1914. Ludwig Pick described the pathology of the disease in a series of papers in the 1930s.

It is an autosomal recessive disorder (OMIM:257200, OMIM:607616) due to the deficit of lysosomal acid-Sphingomyelinase (ASM; E.C. 3.1.4.12), that converts sphingomyelin to ceramide and phosphorylcholine. It results in intracellular accumulation of sphingomyelin mainly in the monocyte/macrophage system.

NP is a panethnic disorder with an estimated global incidence of 1:100000 live births. The incidence of NP is higher in populations as Northern Africans, Arabs or Ashkenazi Jewish descent [63].

The disease can be divided into two distinct subtypes based on their rate of progression and involvement of the central nervous system: NP-A represents the neuropathic and most severe form, characterised by hepatosplenomegaly,

psychomotor retardation, recurrent respiratory infections and subsequent death during or before the third decade of life. NP-B is typically later in onset and milder in manifestation, with no neurologic involvement [64,65].

Molecular aspects

ASM gene (designated SMPD1), composed of 6 exons, is located on human chromosome 11p15.1-11p15.4 (Schuchman and Desnick, 2001) and encodes for a 629 aa protein. Several mutations have been identified to cause the disease, including single base substitutions, small deletions/insertions and one splice-site alteration [66].

(<http://www.uniprot.org/uniprot/P17405>)

Treatment

At the moment therapies for NP are mostly supportive. Only few patients with NP-B underwent successful bone marrow transplantation [67]. Enzyme replacement therapy for NP-B has concluded its Phase 1 clinical trial in 2009.

1.3.6 Mucopolysaccharidosis type I or Hurler Syndrome

The syndrome is named from the German pediatrician Gertrud Hurler who first described the disease in 1919. It is an autosomic recessive disorder (OMIM 252800) caused by the deficit of the enzyme α -Iduronidase (EC 3.2.1.76) responsible of the hydrolysis of unsulfated α -L-iduronosidic linkages in dermatan and eparan sulfate with consequent accumulation of these glycosaminoglycans in lysosomes. MPS I has an estimated incidence of 1 case per 100000 live births and the attenuated type represents about 20% of the total MPS I population.

MPS I includes separate diseases on the basis of clinical presentation: Hurler, Hurler-Scheie, and Scheie Syndromes.

Hurler is the most severe form; patients develop symptoms in early childhood and have marked cognitive delay, hepatosplenomegaly, heart problems, corneal clouding, airway obstruction and severe joint restriction [68]; they usually don't survive longer than the first decade of life.

Hurler-Scheie is the intermediary form, with a similar clinical spectrum, but a slower rate of progression.

Finally, Scheie's Syndrome is the mildest form, patients present less extensive disease and a potentially normal life span.

The difference in severity is due primarily to the effect of various mutations, some of which permit residual enzyme activity [69].

Molecular aspects

IDUA gene consists of 19 kb and is located on chromosome 4, locus 4p16.3. The gene comprises 14 exons and encodes a precursor protein of 653 amino acids. To date, more than 100 pathogenic mutations and numerous nonpathogenic sequence variants have been reported [70].

(<http://www.uniprot.org/uniprot/P35475>).

Treatment

Bone marrow transplantation, especially if performed early in life, could be an effective treatment for patients with Hurler's syndrome, even if the morbidity and mortality of the procedure and the need for matched donor marrow limit its usefulness.

Enzyme-replacement therapy is available with laronidase (human recombinant α -L-iduronidase, Aldurazyme) [71].

Gene therapy has been assayed with retroviral, lentiviral and adeno-associated virus vectors, also non viral vectors have been evaluated to deliver the iduronidase gene. By now treatment of the mouse, dog, and cat models of MPS I are in trial [72].

1.4 Mass Spectrometry

The origin of Mass Spectrometry (MS) dates back to 1898 with the discovery by W Wien, that beams of charged particles could be deflected by a magnetic field; this concept mainly developed thanks to the work of two physicist, Thomson and Aston, that studied the properties of canal rays (positive ions) in a vacuum tube, exposed to electrostatic and magnetic field: ions could be deflected through small angles according their mass to charge ratio and detected with a photographic plate [73].

It took more than 30 years to spread the spectrometry technology out of the academic field where it was applied mainly to isotopes identification, to measure their relative abundance and their atomic mass (with an accuracy of 1 ppm). Those findings throw the basis for an ever-increasing number of mass spectrometry applications in science and technology.

So what is Mass Spectrometry?

“Mass spectrometry is the art of measuring atoms and molecules to determine their molecular weight. Such mass or weight information is sometimes sufficient, frequently necessary, and always useful in determining the identity of a species. To practice this art one puts charge on the molecules of interest, i.e., the analyte, then measures how the trajectories of the resulting ions respond in vacuum to various combinations of electric and magnetic fields.

Clearly, the sine qua non of such a method is the conversion of neutral analyte molecules into ions. For small and simple species the ionization is readily carried by gas-phase encounters between the neutral molecules and electrons, photons, or other ions. In recent years, the efforts of many investigators have led to new techniques for producing ions of species too large and complex to be vaporized without substantial, even catastrophic, decomposition.” (John B. Fenn, the originator of electrospray ionization for biomolecules and the 2002 Nobel Laureate in Chemistry).

1.4.1 Uses of mass spectrometry

The mass spectrometer technique could be used both for qualitative and quantitative aims, such as:

- Identification of unknown compounds
- Determining the isotopic composition of elements in a compound
- Determining the structure of a compound by observing its fragmentation
- Quantifying the amount of a compound in a sample using carefully designed methods
- Determining the molecular weight of compounds
- Studying the fundamentals of gas phase in ion chemistry
- Determining other physical, chemical or biological properties or compounds
- Determining intermolecular interactions

1.4.2 Principle of mass spectrometry

All the mass spectrometers are made up of four standard elements (Fig.1):

- Sample inlet
- Ionization source
- Mass analyzer
- Ion detector

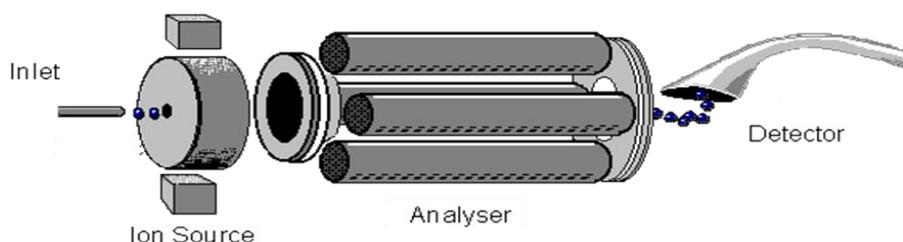


Fig.1 Basic elements of a mass spectrometer

The samples, in liquid, solid or gaseous state, are injected in the spectrometer and ionized.

Resulting ions are electro-statically propelled to the analyzer, separated according their mass to charge ratio (m/z) and then collected by a detector that converts the ion energy into electric signals, witch intensity will be proportional to the number of ions. Several pumps are used to strictly maintain all the machinery in an high vacuum condition ($\sim 10^{-7}$) [74,75].

a. Sample Inlet

Samples could be injected directly on a probe or plate in the ionization region of the mass spectrometer typically through a vacuum interlock, that allows samples at atmospheric pressure (~ 760 torr) to be introduced in a high vacuum area (\sim

10^{-6}). Otherwise they could be injected by direct infusion through a capillary or a capillary column as a gas or in solution.

The need to analyse complex mixture and to obtain a spectra of a single component, led to the combination of separation technique with mass analysis: gas chromatography (GC) was first coupled with a MS in the 60s to separate different components in gaseous mixture prior mass analysis, but it is unsuitable for non volatile or thermally fragile molecules [76]. Instead, the liquid chromatography (LC) can safely separate a very wide range of organic compound, but it was not used until the 90s since the ionization techniques were unable to handle the continuous flow of LC [77]. Nowadays High Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography (UPLC), Capillary Electrophoresis (CE) and Supercritical Fluid Chromatography (SFC) are currently used.

Sample ionization

According on samples nature and on witch pattern of information you are interested in, several ionization techniques could be used; they include protonation, deprotonation, electron ejection, electron capture, cationization, or by transferring a charged molecule from a condensed phase to the gas phase.

b. Ionization source

Prior to 1980s electron ionization (EI) was the primary ionization source for mass analysis. The main limit, that induced lots of scientists to develop a new generation of ionization techniques, was that EI can not be used with high mass compound. That excludes the majority of bio-organic molecules.

A good ionization source must satisfy several requirements: it must have an high ionization efficiency and an high transmission coefficient, it has to produce monoenergetic ion beam, guarantee an high signal to noise ratio and a low memory effect [75].

The available methods are listed in Tab.1

Ionization Source	Acronym	Event	Mass range
Electrospray ionization	ESI	Evaporation of charged droplets	less than 200,000 Da
Nano-electrospray ionization	nanoESI	Evaporation of charged droplets	less than 1000 Da
Atmospheric pressure chemical ionization	APCI	Corona discharge and proton transfer	less than 2000 Da
Matrix-assisted laser desorption/ionization	MALDI	Photon absorption/proton transfer	less than 500,000
Desorption/ionization on silicon	DIOS	Photon absorption/proton transfer	less than 18,000 Da
Fast atom/ion bombardment	FAB	Ion desorption/proton transfer	300-6000 Da
Electron ionization	EI	Electron beam/electron transfer	less than 1,000 Da
Chemical ionization	CI	Proton transfer	less than 1,000 Da

Tab.1 Ionization sources for mass spectrometry

They are mainly divided in “hard” and “soft” according to the level of energy involved during the ionization process, that determines the degree of molecular fragmentation.

MALDI and ESI are now the most common ionization sources for biomolecular mass spectrometry, offering excellent mass range and sensitivity.

c. Mass analyzer

In the most general terms a mass analyzer uses electric and magnetic field to separate and measure gas phase ions (with respect to their mass to charge ratio); the force applied causes an acceleration that is mass (Newton’s second law) and ionic charge (Lorentz force law) dependent.

The first mass analyzer was made in the early 1900’s and it separates ions according to their radius of curvature through a magnetic field.

In the last years the design of mass analyzer has been in continuum progress to follow the rapid evolution of ionization techniques and satisfy the demand of analyzing a wide range of bio molecular ions with part per million mass accuracy and sub-femtomole sensitivity; the most common mass analyzers are:

- Magnetic sector
- Quadrupole
- Ion traps (Quadrupole ion trap or Paul trap and Static trap or Penning trap)
- Fourier transform Ion Cyclotron Resonance (FT-ICR)
- Time of flight (TOF)
- Quad-TOF mass analyzer

Multiple mass analysis (MS/MS, MSⁿ)

This is the ability of the analyzer to separate different molecular ions, fragment selected ions, and then measure the masses of the obtained fragments. This type of data is used for sequencing (peptides and sugars), structural elucidation and analyte identification through fragment fingerprinting.

Tandem mass analysis

Tandem mass analysis could be performed in space or in time.

- *Separation in space.*

It is made by consecutive analyzers physically separated and distinct, although there is a physical connection between the elements to maintain high vacuum.

The most common tandem mass spectrometer is a triple quadrupole, in which two quadrupole (Q1 and Q3) work as mass filter and one (Q2) as collision cell.

Four most common types of MS/MS experiment are:

- 1) Product ion scan: the precursor ion is selected in Q1, fragmented in Q2 through collision (collision induced dissociation CID) with gas molecules (Argon or Helium), and then all fragments are measured by scanning in Q3
- 2) Precursor ion scan: ions are scanned in Q1, fragmented in Q2 and only a particular fragment ion is measured in Q3. The result is a spectrum of precursor ions that produce a particular product ion.
- 3) Neutral loss scan: Q1 works as in (2), but this time measured all ions that have undergone a particular mass loss. In this case the spectrum represents all the precursor ions that undergo a particular neutral loss.
- 4) Selected reaction monitoring(SRN or MRM): Both Q1 and Q3 are set to a selected

Triple quadrupole and Q-ToF instruments tend to produce more energetic CID with more fragmentation, but less operator control

- *Separation in time*

It is accomplished with ions trapped in the same place, with multiple separation steps taking place over time. Ion traps or FT-MS instruments can be used for such an analysis. Ion-trap and FT-MS instruments allow for the most control over CID, but also tend to produce less energetic reactions

The main advantage of separation in time is that it allow multistage fragmentation experiments (MS^n), essential for structural elucidation studies since it allows the re-fragmentation of product ions.

d. Ion detector

All mass analyzer, except the FT-ICR, that is a combined mass analyzer and detector, need accurate and reliable ion detection.

The choice of the detector depends on the design of the instrument and on the type of the experiment.

The first ion detector dates back in the days of Thomson and Aston and consists of photographic plates placed at the end of the analyzer. It is based on the fact that all ions of a given m/z would impact at the same place on the photographic plate making a spot, and that its intensity is proportional to the darkness of the spot.

Nowadays different types of detector are in use:

- Faraday cup or cylinder
- The electron multiplier
- The photomultiplier or scintillation counter
- Array detector
- Charge (or inductive) detector

Performance characteristics

- **Accuracy**

It is largely a function of an instrument's stability and resolution and it can be expresses in percentage or in ppm (part per million). It represents the capability with which the analyzer can accurately provide m/z information.

- **Resolution**

It is the ability to distinguish between ions of different mass to charge ratios.

The most common definition of resolution is:

$$R = M/\Delta M$$

Where M is the m/z and ΔM is the width at half maximum.

To some extent the accuracy is determined by the resolving power, for example as regard the instrument ability in resolve isotopes; if not, it will generate a broad peak with the centre representing the average mass.

- **Mass range**

This is the m/z range of the mass analyzer. For instance, quadrupole analyzers typically scan up to m/z 3000. A magnetic sector analyzer typically scans up to m/z 10000 and time-of-flight analyzers have virtually unlimited m/z range.

More details on the mass spectrometry technology are reported in **Appendix A**.

1.4.3 Biological applications of mass spectrometry

History

The first application of mass spectrometry to biology dates back to the '40s, when heavy stable isotopes were used as tracers to study processes such as CO_2 production in animals.

At the beginning only volatile compounds could be analyzed, but improvements in the range of detectable masses and in the types of samples that could be vaporized made possible the study of organic compounds.

Few years later, MS was applied to peptides and oligonucleotides sequencing, and in 1962 it was used to study the structure of nucleotides [78].

In 1983 Donike et al. proposed the testosterone/epitestosterone (T/E) excretion ratio measurement with gas chromatography-mass spectrometry (GC-MS), that was first employed in Olympic doping control during the 1984 Los Angeles Olympic Games [79].

In the '80s, the introduction of the fast atom bombardment ionization method allowed ionization of larger molecules such as proteins [80] that further improved with the advent of electrospray ionization (ESI) by Fenn and coworkers in 1988 [81]; moreover ESI could be easily connected to an on-line liquid chromatography (LC), which made possible the analysis of complex mixtures.

Protein structure was first studied by MS in '90s [82], thanks to the advent of MALDI (matrix-assisted laser desorption/ionization) technology, and the peptide mass fingerprinting was developed immediately later for protein identification.

Isotope-coded affinity tags (ICAT), at the end of the 90s, made possible the quantification of individual proteins in a complex mixture. This technology allowed the simultaneous comparison of the expression levels of all proteins in cells grown in different media [83].

The first clinical applications of mass spectrometry dates back to 1966, when the use of gas chromatography coupled to mass spectrometry for identification of organic acidurias in children was reported. Twenty years later tandem mass spectrometry (MS/MS) was introduced into clinical laboratories, mainly because of its enhanced sensitivity and specificity, and it was primarily applied to the evaluation of children at risk of inborn errors of metabolism.

Up to now MS has having an exponential growth in clinical laboratory applications of which pharmacology and proteomics have been the major drivers.

Mass spectrometry for drug monitoring and toxicology studies

Mass spectrometry is widely used for therapeutic drug monitoring (immunosuppressive drugs, antidepressants, etc) and for the detection of illicit pharmacological agents, their metabolites and testosterone and epitestosterone conjugates, especially in anti-doping procedures [84]. Since ESI MS/MS enables the direct determination of the steroid conjugates, the technology facilitates screenings.

Mass spectrometry for diagnostic procedures

LC-MS/MS has rapidly substituted several traditional diagnostic methods, especially those that have tedious specimen preparation protocols or prohibitive interference problems, thanks to its sensibility, reliability and capability to detect low analytes concentration and to make simultaneous analysis.

For example, LC-MS/MS was the gold standard for Methylmalonic acid (MMA) determination in serum, plasma or urine; MMA, that is employed as a reflex test for intermediate serum vitamin B12 concentrations, could not be detected before the advent of mass spectrometry because of its low endogenous concentration and potential interference from other low molecular weight organic acids.

Similarly, LC-MS/MS was the revolution for the diagnosis of some endocrine tumours, since it allows the measurement of plasma free metanephrine that occurs in low nanomolar concentration in a complex matrix. With the advent of

LC-MS/MS all tests performed became more rapid, sensitive and resulted easier to interpret.

In Cushing syndrome, characterized by a chronic state of cortisol excess LC-MS/MS became the only method providing the specificity required for a correct diagnosis, as automated immunoassays lack the sensitivity to detect the low salivary cortisol concentrations [85].

ESI MS/MS can be used to determine urinary sulfatide concentrations to distinguish metachromatic leukodystrophy from pseudodeficiency in those patients with low Arylsulfatase A activity [86]; or for the detection of bile acid metabolites arising from inherited defects in bile acid metabolism [87,88].

Urine oligosaccharides have been determined by MS/MS using their 1-phenyl-3-methyl-5-pyrazolone derivatives [89].

Finally, combination of MALDI-TOF MS-based system with specific software and database of more than 2,000 microorganism species is now routinely being used as a diagnostic tool for microbial identification in many European laboratories because it shortens time to results, reduces costs and increases accuracy compared to conventional biochemical assays [90].

Mass spectrometry and screening

The real revolution of the clinical application of mass spectrometry was in the field of the screening; in fact, starting in the '90s the introduction of tandem mass spectrometry (MS/MS) for population-based newborn screening programs, has enabled to detect an increasing number of metabolic disorders in a single process by using dried blood-spot specimens [91].

MS/MS technology allowed improvements in the analysis of amino acid disorders (PKU, maple syrup urine disease and homocystinuria) among newborns, with a false-positive rate 10-fold lower than the best method previously available [92,].

Successively the metabolic disorder screening panel was expanded by incorporating an acylcarnitine profile (eliminating time-consuming chromatography but maintaining method specificity), which enables the detection of fatty acid oxidation disorders (medium-chain acyl-CoA dehydrogenase [MCAD] deficiency) [93].

Also optimization of the system has facilitated the simultaneous screening for various disorders of organic acid degradation, fatty acid oxidation, the urea cycle and amino acids.

More than 30 inborn errors of metabolism can now be rapidly detected by MS/MS.

A rapid and specific screening method by HPLC ESI MS/MS [94] has been shown to be effective for the analysis of purine nucleoside phosphorylase deficiency, ornithine transcarbamylase deficiency, molybdenum cofactor deficiency, adenylosuccinase deficiency and dihydropyrimidine dehydrogenase deficiency in patients at risk of inherited disorders of purine and pyrimidine metabolism.

Recently, a protocol has been developed for the quantification of hexose mono phosphates in blood spots by negative ion ESI MS/MS [95] which could be applicable to newborn screenings for galactosemia. Screening for congenital adrenal hyperplasia by detection of 17- α -Hydroxyprogesterone in blood spots has also been reported [96], as well as a rapid screening procedure for the diagnosis of Smith-Lemli-Opitz syndrome [97].

For several metabolic disorders, early detection, with screening programs, can result in substantial improvements in health outcomes, and pilots programs in Australia, New England and NY suggested a higher incidence of metabolic disorders compared to clinical diagnosis [98].

In Italy, since 2004, a screening program for nearly 50 metabolic disorders, including LSDs, is conducted with so encouraging results [33] that in 2006 part of the Umbria Region joined the project.

Finally, all commercially available dedicated screening systems are currently based on MALDI-TOF technology, that has been demonstrated to be a superior analytical tool for various screening and diagnostic applications, like SNP genotyping, oligonucleotide quality control or biomarker screening. Measuring an intrinsic property of the analyte molecules (the molecular weight) is neither a need for indirect detection methods (hybridisation, labelling with fluorescent dyes or radioactive isotopes) nor for antibody-based detection in the case of profiling applications in clinical proteomics [99].

AIM OF THE STUDY

Lysosomal storage disorders are a group of about 50 inherited metabolic diseases, with a global incidence of 1:1500-7000 live birth. They are mainly due to the deficit of lysosomal enzymes which leads to the development of complex, severe and progressive disorders.

Few therapeutic approaches are now available, however in most cases, they cannot be really effective, since the correct diagnosis usually occurs when irreversible symptoms have already developed.

Several studies performed in the last few years have shown these pathologies to be more frequent than expected; moreover, analysis on specific groups of patients (affected by cryptogenic stroke or renal failure) has identified some cases of Fabry Disease previously misdiagnosed.

Another relevant point of discussion is the need of a correct determination of LSDs incidence in the population, which will have an important fall-out both on the attention of the medical community to LSDs and on the future plans of the Countries with respect to these disorders.

That considered, together with the great development and the encouraging results of enzymatic analysis on Dried Blood Spot, first performed with fluorimetric tests, and more recently with tandem mass spectrometry, the aim of this work was to set up and to optimize a rapid, reliable, sensitive and non invasive method for the multiplex detection of enzyme activity in a high number of samples.

Main aim of this work was focused on the reproducibility and reliability of the protocol, underlying the most critical steps that need to be optimized; in addition, an evaluation of the feasibility of a newborn screening project was made.

The second purpose was to improve the protocol, reducing at a minimum chances to introduce biases during the preparation of samples, and optimizing all the analytical parameters for MSMS analysis.

Main challenge of this study was to define a method that could, first of all, be used for a newborn screening program, also able to guarantee an as low as possible false positive rate, being this last one of the most controversial point in the decision to introduce or not LSDs in the panel of screened disorders.

Moreover the set up protocol could be proposed to screen not only newborns, but also specific high risk populations, or populations presenting with particular clinical manifestations, as nephropathic or cardiopathic subjects, therefore helping to possibly correctly identify under/misdiagnosed patients.

Finally, being a rapid method for the detection of enzymes availability in blood, it could be applied to patients follow up, allowing an innovative and more personalized approach in the treatment of LSDs.

2. MATERIALS AND METHODS

- **Samples**

- ✓ Specimens from anonymous newborn were collected at the Wadsworth Center, Albany, New York and at the Szeged Hospital, Hungary. Blood sampling typically occurred between 48 and 72 h of life. DBS were shipped at RT and then stored desiccated at $-20\text{ }^{\circ}\text{C}$ until analysis.
- ✓ Blood of healthy adults have been used. The blood was anonymously collected in EDTA tubes, immediately spotted on to filter paper (Whatman 903, Whatman plc, Kent, UK www.whatman.com, 60 μL /spot) and dried at room temperature. DBS were then stored desiccated at $-20\text{ }^{\circ}\text{C}$ until analysis.
- ✓ Samples from patients with a confirmed diagnosis of type I Gaucher Disease and Niemann Pick A/B Disease were obtained from Genzyme Inc. Framingham, USA.
- ✓ Samples from patients with a confirmed diagnosis of Pompe and Fabry Disease were obtained from our clinical unit and Florence, respectively

For all samples blood was drawn following written, informed consent.

- ✓ Control samples (Low, Medium, High activity) have been supplied by the Center of Disease Control and Prevention (CDC), Atlanta, USA

- **Reagents**

Vials of substrate and internal standard for six LSDs (Pompe, Fabry, Gaucher, Niemann Pick A/B, Krabbe and Mucopolysaccharidosis type I) were provided by the CDC of Atlanta, and have been resuspended in specific solutions (Cocktails), according to CDC instructions.

✓ Gaucher

12 μmol ABG substrate D-Glucosyl- β 1-1'-N-dodecanoyl-D-erythro-sphingosine [C12-glucocerebroside (C₃₆H₆₉NO₈)] (molecular weight of 643.9 Da) and 0.23 μmol ABG internal standard N-myristoyl-D-erythro-sphingosine [C14-ceramide (C₃₂H₆₃NO₃)] (molecular weight of 509.8 Da) in a molar ratio of approximately 50:1

Cocktail:

ABG assay cocktail was prepared by the sequential addition of 15.6 ml of Gaucher buffer (0.715 mol/L Sodium Phosphate Monobasic (NaH₂PO₄) (Sigma, MO, USA, www.sigmaaldrich.com) and 0.358 mol/L Sodium Citrate Tribasic Dihydrate (C₆H₅Na₃O₇) (Merck; pH 5.1) and 2.4 ml of sodium taurocholate (120 g/L in water) (Sigma). The vial was vortexed until reagents were completely dissolved and then stored at -20 °C. The final ABG assay cocktail contained 0.67 mmol/L β -Glucocerebrosidase substrate, 13.05 $\mu\text{mol/L}$ β -Glucocerebrosidase internal standard and 0.029 mmol/mL sodium Taurocholate.

✓ Niemann Pick:

3.37 mg (5.9 μmol) of ASM substrate, N-Hexanoyl-D-erythro-sphingosylphosphorylcholine [C6-sphingomyelin (C₂₉H₅₉N₂O₆P)], and 0.04 mg (0.1 μmol) of NP internal standard, N-butyroyl-D-erythro-sphingosine [C4-ceramide (C₂₂H₄₃NO₃)], in a molar ratio of approximately 50:1.

Cocktail:

ASM assay cocktail was prepared by the sequential addition of 150 μl of detergent solution (120 g/L Sodium Taurocholate in water) and 17.85 ml of buffer (930 mol/L Sodium Acetate + 0.604 mmol/L Zinc Chloride, pH 5.7). Reagents were completely dissolved and vials stored at -20°C. The final NP assay cocktail contained 0.33 mmol/L substrate, 6.67 $\mu\text{mol/L}$ internal standard and 1.8 mmol/L Sodium Taurocholate.

✓ Pompe:

7.9 mg (11.9 μmol) of GAA substrate, (7-benzoylamino-heptyl)-{2-[4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yoxy)-phenylcarbamoyl]-ethyl}-carbamic acid tert butyl ester ($\text{C}_{34}\text{H}_{49}\text{N}_3\text{O}_{10}$), and 0.06 mg (0.11 μmol) of Internal Standard (7-d5-benzoylamino-heptyl)-[2-(4-hydroxy-phenylcarbamoyl)-ethyl]-carbamic acid tert butyl ester ($\text{C}_{28}\text{H}_{34}\text{N}_5\text{O}_5\text{D}_5$), in a molar ratio of approximately 100:1.

Cocktail:

GAA assay cocktail was prepared by the sequential addition of 0.306 ml of acarbose (5.165 mg/ml), 15.9 ml of buffer (0.34M Sodium Phosphate, 0.17 M Sodium Citrate, pH 4.0) and 1.8 ml of CHAPS (100g/l). Reagents were completely dissolved and vials stored at -20°C . The final GAA assay cocktail contained 0.661 mmol/L substrate, 6.1 $\mu\text{mol/L}$ internal standard, 0.136 mmol/L Acarbose and 0.016 mmol/ml CHAPS.

✓ Fabry:

38.72 mg (60 μmol) of GLA substrate, (6-benzoylamino-hexyl)-{2-[4-(3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-phenylcarbamoyl]-ethyl}-carbamic acid tert butyl ester ($\text{C}_{33}\text{H}_{47}\text{N}_3\text{O}_{10}$), and 0.06 mg (0.12 μmol) of Internal Standard (6-d5-benzoylamino-hexyl)-[2-(4-hydroxy-phenylcarbamoyl)-ethyl]-carbamic acid tert butyl ester ($\text{C}_{27}\text{H}_{32}\text{N}_5\text{O}_5\text{D}_5$), in a molar ratio of approximately 500:1.

Cocktail:

GLA assay cocktail was prepared by the sequential addition of 450 μl of Sodium Taurocholate (120 g/L), 14.67 ml of buffer (0.174M Sodium Acetate, pH 4.6) and 2.88 ml of N-acetyl-galactosamine (1M). Reagents were completely dissolved and vials stored at -20°C . The final GLA assay cocktail contained 3.33 mmol/L substrate, 6.66 $\mu\text{mol/L}$ internal standard, 5.5 mmol/L Sodium Taurocholate and 0.159 mmol/ml N-acetyl-galactosamine.

✓ Krabbe:

10.57 mg (17.9 μmol) of GALC substrate, D-Galactosyl- β 1-1'-octanoyl-D-erythro-sphingosine [C8-galactosylceramide ($\text{C}_{32}\text{H}_{61}\text{NO}_8$), and 0.05 mg (0.11 μmol) of Internal Standard N-decanoyl-D-erythro-sphingosine [C10-ceramide ($\text{C}_{28}\text{H}_{55}\text{NO}_3$), in a molar ratio of approximately 150:1.

Cocktail:

GALC assay cocktail was prepared by the sequential addition of 1.8 ml of detergent (96 g/L Sodium Taurocholate, 12 g/L oleic acid) and 16.2 ml of buffer (0.2M Phosphate and 0.1M Citrate, pH 4.4). Reagents were completely dissolved and vials stored at -20°C . The final GALC assay cocktail contained 0.994 mmol/L substrate, 6.1 $\mu\text{mol/L}$ internal standard, 0.017 mmol/ml Sodium Taurocholate and 4.2 mmol/L Oleic Acid.

✓ MPS I

17 mg (30 μmol) of IDUA substrate, (7-(1-Iduronic acid)-oxycoumarin-4-acetic acid-(1',4'-N-boc-diaminobutane)-amide) ($\text{C}_{26}\text{H}_{34}\text{N}_2\text{O}_{12}$), and 0.08 mg (0.212 μmol) of Internal Standard, (7-hydroxycoumarin-4-acetic acid-(1',3'-N-boc-diaminopropane)-amide) ($\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_6$), in a molar ratio of approximately 150:1.

Cocktail:

IDUA assay cocktail was prepared by the sequential addition of 0.5 ml D-saccharid acid 1,4 lactone monohydrate (0.63 mg/ml) and 17.5 ml buffer (0.112 M Sodium Formiate, 0.158 M formic acid, pH 3.6). Reagents were completely dissolved and vials stored at -20°C . The final IDUA assay cocktail contained 1.666 mmol/L substrate, 11 $\mu\text{mol/L}$ internal standard and 0.83 $\mu\text{mol/ml}$ Lactone.

2.1 Experiments performed at the Vienna Centogene Unit

During the first year and a half of my PhD period I worked at the Centogene Laboratory of the Vienna Biocenter.

Experimental plan

2.1.1 Gaucher and Niemann Pick A/B assays set up

The first step of the work was the β -Glucosidase (Gaucher) and Acid-Sphingomyelinase (Niemann Pick A/B) assays set up and their inclusion in one multiplex test together with Pompe, Fabry and Krabbe assays, that were already performed in this laboratory. β -Glucosidase (ABG) and Acid-Sphingomyelinase (ASM) stability in DBS of an healthy adult control, have been monitored for three months at different storage temperatures (-80°C, -20 °C, 4 °C, room temperature and 37 °C).

Blood from an healthy adult was used to analyze the effect of hematocrit and location of the punch on ABG and ASM activity. EDTA blood was spun down and the cellular component was mixed with the plasma at different ratios to generate six groups of DBS with different hematocrit concentrations (30%, 40%, 50%, 60%, 70% and 80%). Enzyme activities in 6 peripheral and 6 central punches of the same DBS were detected.

The same healthy adult sample stored at -20°C was used to carry out the assay variability; for the inter-assay repeatability four punches from different DBS were measured on 7 different days, while for the intra-assay variability ten punches obtained from the same DBS were analyzed simultaneously.

Moreover, 12 punches of the same sample and 12 blank punches used to investigate possible carryover problems were always used.

For Niemann Pick A/B, Gaucher, Pompe and Fabry Disease 2088 anonymous newborn samples were analyzed to generate newborn reference ranges; then 10 previously diagnosed cases with Gaucher Disease and 10 with confirmed NiemannPick A/B Disease were tested.

2.1.2 Activity comparison for β -Glucosidase, α -Galattosidase and α -Glucosidase

An activity comparison study was performed on three enzymes (β -Glucosidase, α -Galattosidase and α -Glucosidase) between two different laboratories; 435 newborn samples were analyzed in parallel in Vienna and at the Wadsworth center of NY. Then, to discriminate if differences were due to the preparative steps or to the spectrometric analysis, all plates from each laboratory were dried out and sent to the other laboratory to repeat the MSMS analysis.

2.1.3 Newborn screening pilot project

A newborn screening pilot project was conducted on 10000 anonymous samples collected at the Szeged Hospital (Hungary) for four lysosomal storage disorders (Pompe, Fabry, Gaucher and Niemann Pick A/B).

Each sample was first tested singly, then, samples with activity lower than the fixed cutoffs were retested in duplicate. DBS of samples confirmed at the second test, were sent to a third laboratory (Centogene in Rostock) for the molecular analysis.

Sample preparation

3.2 mm spots from the DBS were punched into 96-well plates (Greiner Bio-one, Frickenhausen, Germany; www.greinerbioone.com) using a Wallac DBS Puncher (Perkin Elmer, Massachusetts, USA; www.perkinelmer.com).

100 μ L of extraction buffer (20 mmol/L potassium phosphate solution (pH 7.1; Merck, New Jersey, USA; www.merck.com) were added to each well, mixed gently and plates were sealed with a siliconer plate sealer. Samples were extracted at 37°C and 750 rpm with shaking for 1 h in a Heidolph incubator (Incubator 1000, Titramax 1000). After incubation, 10 μ L extract was transferred into a new plate containing 15 μ L assay cocktail in each well. If all the five enzymes are tested simultaneously, five different plates are needed, one for each test; punches for Krabbe assay do not need to be eluted, but 30 μ L of assay cocktail are added directly on the punch.

The plates were sealed and incubated for 21 h at 37°C. The enzyme reactions were quenched by adding 100 μ L of 1:1 ethylacetate:methanol (Merck; BDH Prolabo Chemicals, Leicestershire, UK; www.vwr.com) to each well. After mixing, if more than one enzyme was tested, samples were recollected and transferred into one 96-deep-well plate (Brand). For liquid–liquid extraction we added 500 μ L 1:1 ethylacetate:methanol, followed by 400 μ L ethylacetate (Merck) and 400 μ L distilled water (BDH Prolabo Chemicals).

Samples were centrifuged for 3 min at 3000 rpm. Then, 300 μ L of the upper phase were transferred into a new 96-well plate (Greiner Bio-one) and dried using a Minivap (Porvair Science, Leatherhead, UK; www.porvair-sciences.com). Samples were reconstituted in 100 μ L of 1:19 methanol:ethylacetate and transferred into a 96-well filter plate (Pall Corporations, Port Washington, New

York, USA; www.pall.com) containing silica gel 60 (Merck) to remove any impurity, salts and residual reagents. Resin was washed twice with 400 μ L of 1:19 methanol:ethylacetate. Samples and washes were collected into a 96-deep well plate and dried. Samples were reconstituted with 200 μ L of 80:20:0.01 acetonitrile:water:formic acid (Merck; BDH Prolabo Chemicals) prior to analysis with MS/MS. Each plate contained 5 blank filter papers, 2 positive control samples (LSD-base pool) and two negative control samples (pooled cord blood).

Mass Analyzer

An API 3000 triple–quadruple MS/MS (PE Sciex, Perkin Elmer, Massachusetts, USA; www.perkinelmer.com) in positive ion mode and analyst v. 1.5 software (Applied Biosystems, MDS Sciex, Ontario, Canada) were used for analysis.

The analytes of interests are detected with a Multiple Reaction Monitoring (MRM) approach: in this case the first quadrupole acts as a mass filter for the ions of interest (the precursors), those ions pass then into the second quadrupole, a collision chamber in which impacts with gas molecules cause the ion to shatter into characteristic pieces. In the third quadrupole, instead of obtaining full scan ms/ms, where all the possible fragment ions derived from the precursor are analyzed, only a small number of specific fragment ions (transition ions) are detected.

This allows to obtain the highest sensitivity and selectivity. MS/MS parameters were optimized and reported in Tab. 2:

Analyte	Time (msec)	Declustering Potential (V)	Focusing Potential (V)	Entrance Potential (V)	Collision Energy (V)	Collision Cell Exit Potential(V)	Source temperature (°C)	Ion spray voltage (V)
ASM-IS	200	20	250	10	25	15	150	5500
ASM-P	200	20	250	10	30	15	150	5500
GALC-P	200	45	250	12	28	15	150	5500
GALC-IS	200	45	250	12	28	15	150	5500
ABG-P	200	45	250	12	45	15	150	5500
ABG-IS	200	45	250	12	45	15	150	5500
GLA-P	200	45	250	12	20	15	150	5500
GLA-IS	200	45	250	12	15	15	150	5500
GAA-P	200	45	250	12	21	15	150	5500
GAA-IS	200	45	250	12	15	15	150	5500

Tab.2 Analytical parameter for MS/MS analysis

Those parameters give the highest sensitivity for transitions:

m/z 370.6>264.3 (ASM-IS)

m/z 398.6>264.3 (ASM-P)

m/z 426.5>264.3 (GALC-P)

m/z 454.5>264.3 (GALC-IS)

m/z 482.6>264.3 (ABG-P)

m/z 510.6>264.3 (ABG-IS)

m/z 484.4>384.4 (GLA-P)

m/z 489.4>389.4 (GLA-IS)

m/z 498.4>398.4 (GAA-P)

m/z 503.4>403.4 (GAA-IS)

100% Acetonitrile was used as MS/MS wash solution.

Activity calculation

Enzyme activity was expressed as $\mu\text{mol/h/L}$, and calculation were made according to the formula:

$$\text{Activity: } (P/IS) \cdot IS_{\text{nmol}} \cdot EF \cdot 1000 / T / V_{\mu\text{l}} / RF_{(P/IS)}$$

Where:

(P/IS) is the ratio between Product and Internal Standard peak areas

IS_{nmol} is the amount of IS in assay solution; it is 0.1 for ASM, GLA and GAA; 0,2 for GALC and ABG; 0.35 for IDUA

EF is the elution factor and corresponds to 8

T is the incubation time for the enzymatic reaction, from 20 to 24 hours

V is the amount of blood in the 3.2 mm punch, and it is 3.1 μl

RF is the response factor of product to IS

The response factor was calculate periodically to monitor the Tandem Mass response, using several known concentration of P/IS (0-0,05-0,1-0,5-1-2-5).

In **Appendix B** some examples of chromatograms are shown and precisely one blank sample, a low activity sample (CDC control sample) and a sample with normal activity.

2.2 Experiments performed at the Padova Unit

The second part of my PhD project has been conducted in Padova in collaboration with the Mass Spectrometry Laboratory of the Pediatric Department.

The five assays performed in Vienna have been reproduced in Padova with the addition of the α -L-Iduronidase test for the Mucopolysaccharidosis type I (MPSI). This last assay could be made on the same DBS used for the other enzymatic tests, since the same elution buffer is needed.

First, the protocol has been modified reducing the preparative part. After the plates were incubated with enzymes cocktails for 22h at 37°C shaking, reactions were stopped with Methanol, 1% Formic Acid and re-collected into one vials for each samples. An Ultra Performance Liquid Chromatography (UPLC) step was introduced with a C18 column (Reverse Phase separation). In this way samples undergo directly to UPLC, that is connected online with the mass spectrometer (Waters Alliance Four Ultima, triple quadrupole).

Solvents used for the mass analysis are:

- MeOH + 1% Formic Acid
- H₂O + 1% Formic Acid
- Purge solution: H₂O + 1% Formic Acid
- Wash solution: MeOH + Isopropanol + 0.1% Formic Acid

Successively a perfusion column was introduced before the UPLC (POROS R1 20 μ m, 2.1mmDx30mmL SS 0.1, Applied Biosystems) to obtain a better package and separation of analytes.

The valve with 6 entries has 2 positions and works as follows:

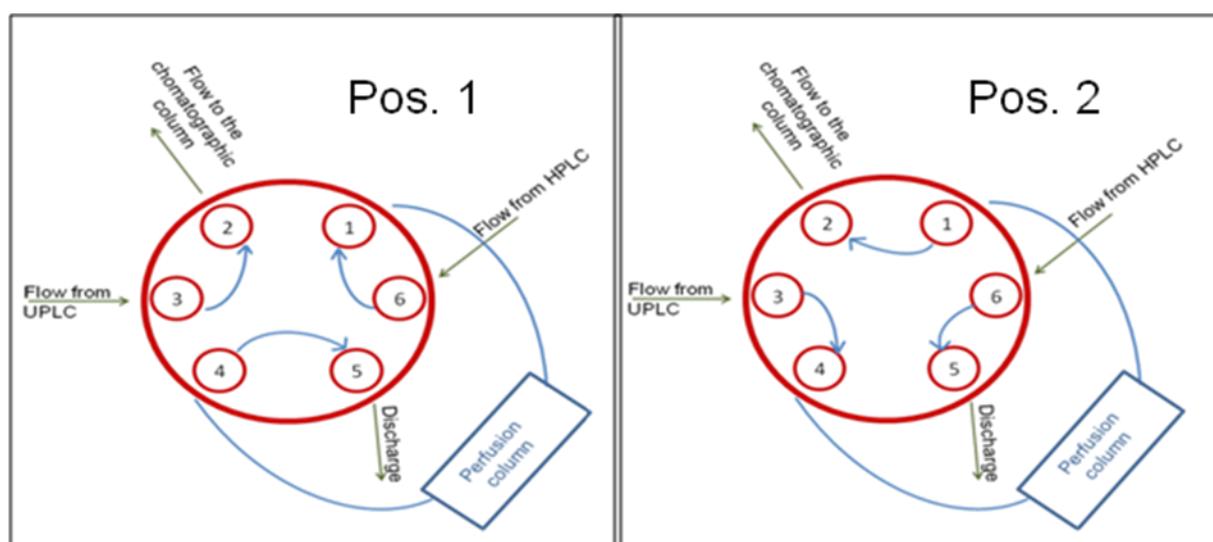


Fig.2 The switching valve mechanism

- 1) The valve is in position 1: the flow with samples comes from the HPLC and enters in position 6; it goes out from position 1 and passes across the perfusion column, the excess goes out in the discharge from position 5. The flow coming from UPLC enters in position 3 and goes directly to the chromatographic column.
- 2) With the activation of the valve (position 2) at 0.5 min, the flow coming from UPLC enters in position 3 and passes through position 4 and then across the perfusion column against the stream of samples entry. In this way it removes and carries analytes into the chromatographic column through position 1 and then 2. The flow from the HPLC goes directly to the discharge.
- 3) With the switching back of the valve the perfusion column is re-equilibrated with the flow coming from the auto sampler to be ready for the next injection. The flow from UPLC goes directly to the chromatographic column that need to be re-equilibrated as well.

Two acquisition functions were defined: the first from time 0 min to 2.5 min; the second from time 2.51 min to time 5.5 min.

Each function includes six channel, for six metabolites:

Function 1: IDUA-P, IDUA-IS, GLA-P, GLA-IS, GAA-P, GAA-IS

Function 2: GALC-P, GALC-IS, ABG-P, ABG-IS, ASM-P, ASM-IS

All chromatographic and spectrometric parameters were optimized (see Results and discussion).

Results were worked out with a Masslynx software.

CDC quality controls (DBS) with low, medium and high activities have been tested, together with a confirmed positive sample for Fabry Disease and for Pompe Disease.

3. RESULTS AND DISCUSSION

3.1 Gaucher and Niemann Pick A/B assays set up

3.1.1 Assay features

The limit of detection was defined as three times the standard deviation of the blank activity values obtained, and was 0.75 $\mu\text{mol/h/L}$ for β -Glucosidase (ABG) assay and 1.13 for acid-Sphingomyelinase (ASM) assay; limit of quantification, settled as five times the standard deviation of the blank activity values obtained using a defined sample volume, was 1.25 $\mu\text{mol/h/L}$ for ABG and 1.88 for ASM.

3.1.2 Intra- and inter-assay variability, carry-over

Ten punches of the same DBS were analyzed simultaneously to assess intra-assay variability while four DBS from the same individual were analyzed during six consecutive days to assess inter-assay variability. Mean, standard deviation and coefficient of variation (CV) are shown in table 3 and 4.

For acid-Sphingomyelinase intra-assay variability is 7.7 while for β -Glucosidase is 9.7; for both enzymes inter-assay variability is approximately 10%. No carryover was observed.

Tab. 3	n	Average activity	SD	CV%	Tab. 4	n	Average activity	SD	CV%
Interassay	4	11	0,9	7,7	Interassay	4	12,7	1,2	9,7
Punch 1	6	10,1	1	9,9	Punch 1	6	11,9	1,6	13,7
Punch 2	6	11,2	1	8,8	Punch 2	6	14,2	2,0	14,0
Punch 3	6	12,1	1,2	9,5	Punch 3	6	11,5	0,9	8,1
Punch 4	6	10,8	1,2	11,6	Punch 4	6	13,3	0,8	6,0
Intraassay	10	10,4	0,9	8,6	Intraassay	10	11,8	0,9	7,7

Tab.3 and Tab. 4 show respectively Acid Sphingomyelinase and β -Glucosidase assay variability

3.1.3 Effect of hematocrit and position of punch

Six peripheral and six central punches of the same DBS were analyzed for each hematocrit level (30%, 40%, 50%, 60%, 70%, and 80%).

Differently from other analytes as acylcarnitine species or amino acids [100], position of the punch within the blood spot does not have any relevant effect on

lysosomal enzymes activity; this was previously demonstrated by Dajnoki and co-workers analyzing the α -Galactosidase and α -Glucosidase enzyme activities [101,28] and it was here confirmed for both acid-Sphingomyelinase and β -Glucosidase.

Then, as ABG activity is mostly derived from leukocytes, increasing hematocrit levels result in enzyme activities linearly increasing ($R^2 = 0.9822$ for peripheral punches, $R^2 = 0.9838$ for central punches), as for GLA and GAA enzymes [101,28]. In such cases parallel measurements of hematocrit may reduce the risk of false positive and/or false negative results. Instead, acid-Sphingomyelinase activity did not change significantly over a range of different hematocrit levels (Fig. 3).

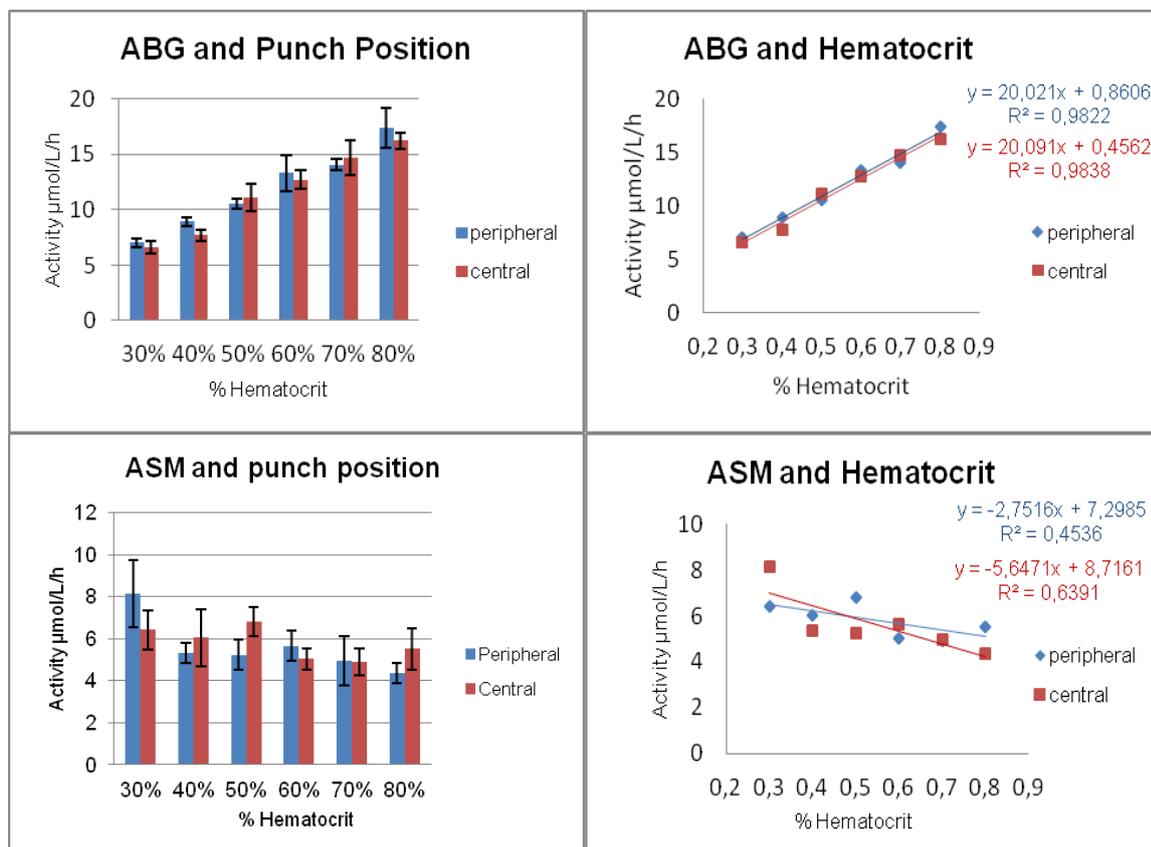


Fig. 3 Correlation between ASM and ABG activity and and punch location and between enzyme activity and hematocrit.

3.1.4 Stability

- *Niemann Pick A/B*

ASM activity in DBS was analyzed in six distinct punches after 7, 14, 21, 28 days and 2 months of storage at 37°C, room temperature, 4°C, -20°C and -80°C. DBS were stored in plastic bags with desiccants. Activities were normalized using ASM activity at -80°C.

- *Gaucher*

ABG stability in DBS at different temperatures (-20 °C to 37 °C) was analyzed for 3 months. Baseline ABG-activity from six different punches of the same DBS (mean: 9.9 µmol/L/h) was defined when DBS were dried. The measurement was repeated at day 1, 4, 7, 14, 21, 28 and at 3 months.

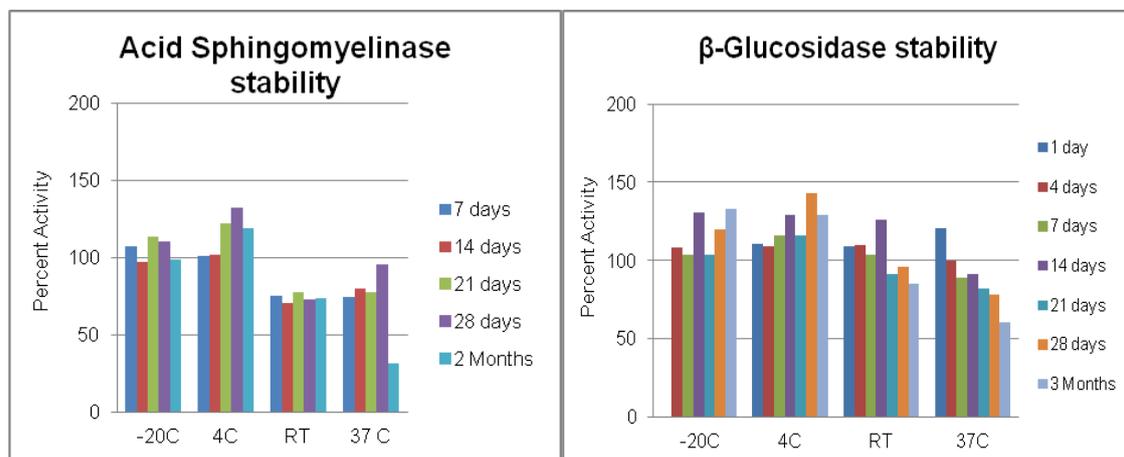


Fig. 4 Acid Sphingomyelinase and beta-glucosidase stability in DBS at different storage temperature

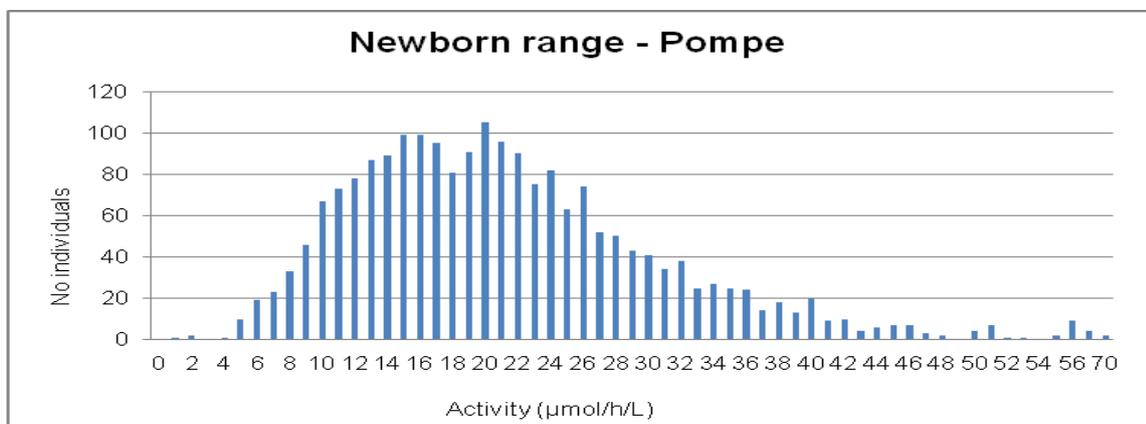
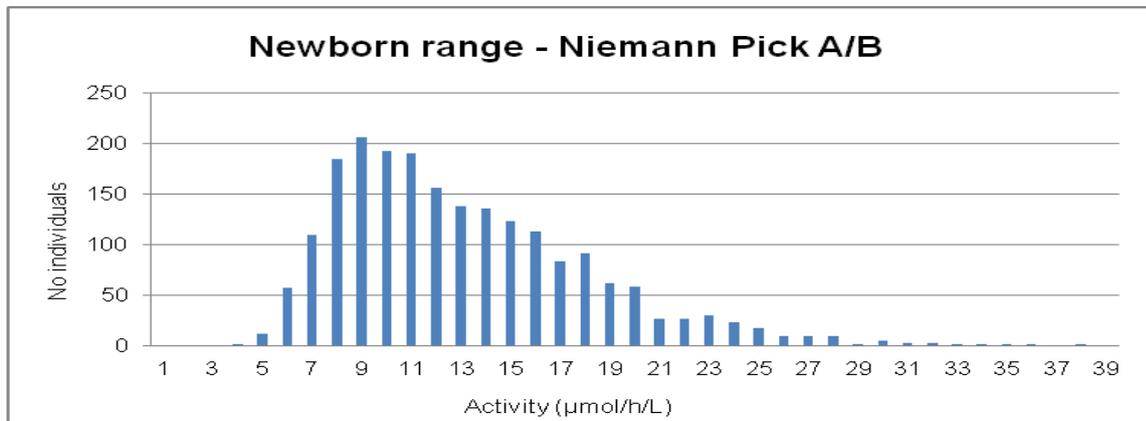
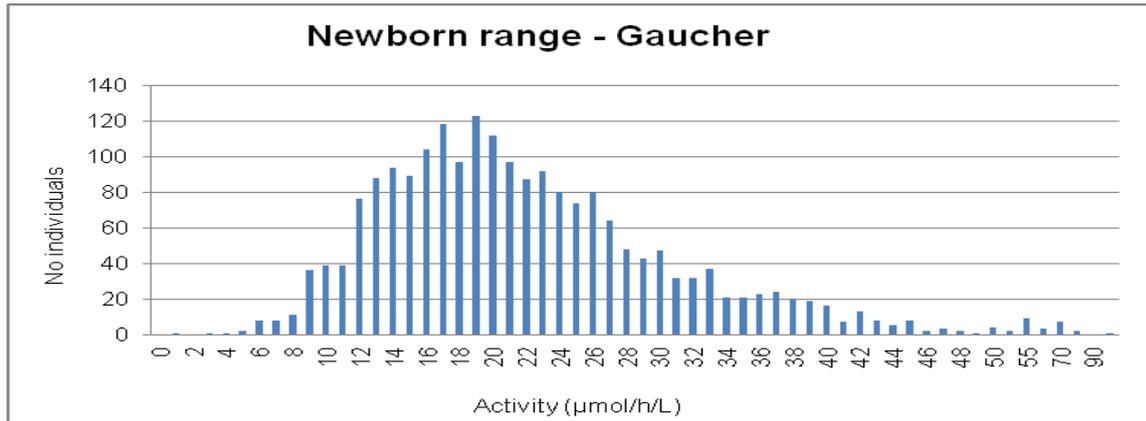
ASM activities were stable for two months when stored at or below 4°C but they decreased by about 20% when stored for 7 days at either room temperature or 37°C. After two months at 37°C only a 30% of activity was detected (Fig.4).

ABG activities, analyzed up to 3 months in sample stored at 4°C to -20°C showed to be stable; at room temperature a 20% reduction of enzyme activity following 3 months of storage was observed, although enzyme activity remained stable for up to 2 week. As expected, ABG activity showed a progressive decline when DBS was stored at 37 °C. Shipment at RT of DBS for ASM and ABG analysis over a longer distance was therefore confirmed to be still feasible. From our evaluation we can conclude that DBS still represent the real innovation for the diagnosis of these pathologies since they allow an easy delivery of the samples

at room temperature, with no problems of deterioration, thus favouring the creation of a few centralized reference laboratories for their analysis [25,102].

3.1.5 Newborn activity ranges

Activity ranges for Gaucher, Niemann Pick A/B, Pompe and Fabry diseases were constituted analyzing 2088 newborn anonymous DBS. Samples presented a normal distribution for all tested enzymes (Fig.5).



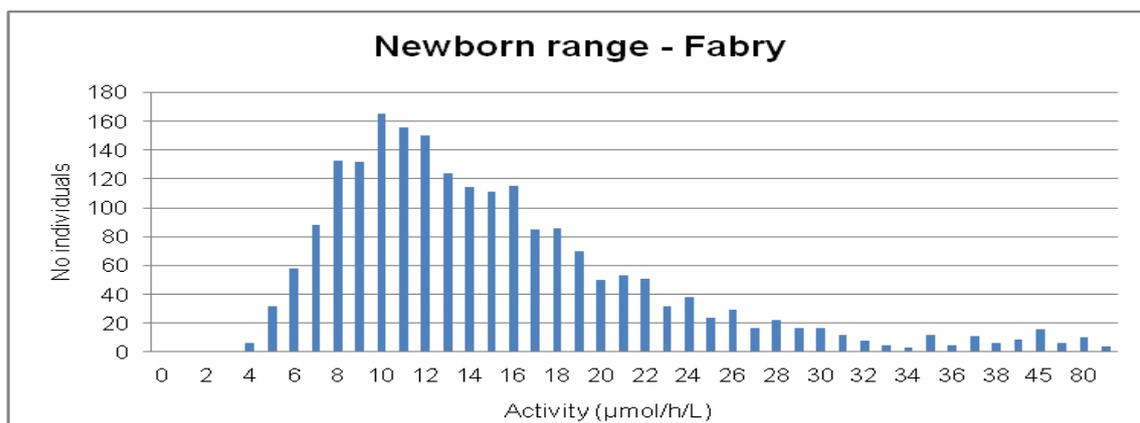


Fig.5 Newborn activities range for Gaucher, Niemann Pick A/B, Pompe and Fabry diseases

Mean, standard deviation, median and 0.5, 1, 99.5 and 99.9 percentiles have been calculated. Values are reported in tab. 5.

	Fabry	Pompe	Gaucher	NP
mean	15,0	21,9	21,8	10,6
st dev	8,8	10,0	10,9	5,1
median	12,9	20,5	19,9	9,5
min	2,6	1,7	0,5	1,2
max	106,8	89,7	181,2	43,9
0,5%	4,2	5,9	5,3	2,9
1,0%	4,6	6,4	7,0	3,2
99,5%	51,6	58,6	62,9	28,1
99,9%	94,3	81,0	139,2	33,9
CUTOFF	3.5	3	4.2	2.5
positives	5	3	3	5
% pos	0.23	0.14	0.14	0.23

Tab.5 Values of activities distribution

The median of enzyme activity in 2088 newborn infants was 12.9 µmol/h/l for GLA, 20.5 for GAA, 19.9 for ABG and 9.5 for ASM.

Considering the 0.5th population percentile and the number of samples that would be repeated, the cut-off value to distinguish between potential disease and non-disease, has been established to be 3.5 µmol/h/l for GLA, 3 for GAA, 4.2 for ABG and 2.5 for ASM.

In such conditions 5 infants were found to have GLA activities below or equal to the cut-off, 3 for GAA, 3 for ABG and 5 for ASM. Unfortunately, due to the

anonymous nature of the study we were unable to confirm these 16 cases (0.76%) that result positive at the first test and would have needed to be re-tested.

The analysis of 2088 samples required less than one month, data analysis included, and no management problems were reported, thus demonstrating the adequacy of the method to test also a high number of samples, which is a basic feature for a possible newborn screening application.

3.1.6 Positive samples

Ten confirmed positive samples of Gaucher Disease and ten of Niemann Pick A/B were analysed and all of them presented an activity lower than the cut-offs previously set (Fig.6).

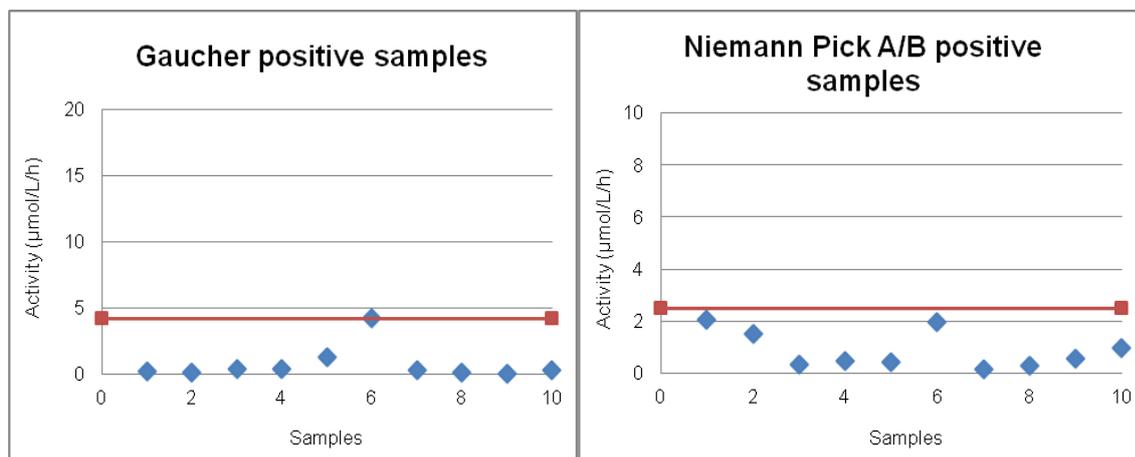


Fig.6 Activities of ten confirmed Gaucher and ten Niemann Pick A/B patients.

3.2 Comparison of β -Glucosidase, α -Galattosidase and α -Glucosidase Activities

B-Glucosidase, α -Galactosidase and α -Glucosidase activities were measured for the 435 newborn samples (five plates). Then, results were compared with those obtained in the Wadsworth Center (NY) on the same newborn samples. Bland-Altman analysis of the data obtained was applied. This approach is based on quantifying the variation in between-method differences for individual patients.

Agreement between two methods of measurement can be represented using the mean of each couple of measurements

$$X_i = \frac{X1_i + X2_i}{2}$$

while the difference between the two observations on the same subjects is indicated by

$$d_i = X1_i - X2_i$$

The Bland-Altman plot is a dispersion graph in which each point is represented by these new couples of values (X_i, d_i) .

The 95% limits of agreement, estimated by mean difference ± 1.96 standard deviation of the differences, provide an interval within which 95% of differences between measurements by the two methods are expected to lie [103].

The concordance correlation coefficient ρ_c [104] was calculated: it evaluates the degree to which pairs of observations fall on the 45° line through the origin. It contains a measurement of precision ρ and accuracy C_b :

$$\rho_c = \rho C_b$$

where

- ρ is the Pearson correlation coefficient, which measures how far each observation deviates from the best-fit line, and is a measure of precision, and
- C_b is a bias correction factor that measures how far the best-fit line deviates from the 45° line through the origin, and is a measure of accuracy.

The comparison shows an acceptable correlation between the two methods, especially related to Fabry (Agreement limits: 1.38, -1.01) and Pompe assays (Limits: 5.42,-9.2). More variability was observed for Gaucher assay (Limits: 10.94, -20.0). The plate to plate comparison showed an homogeneous distribution of results (Fig. 7).

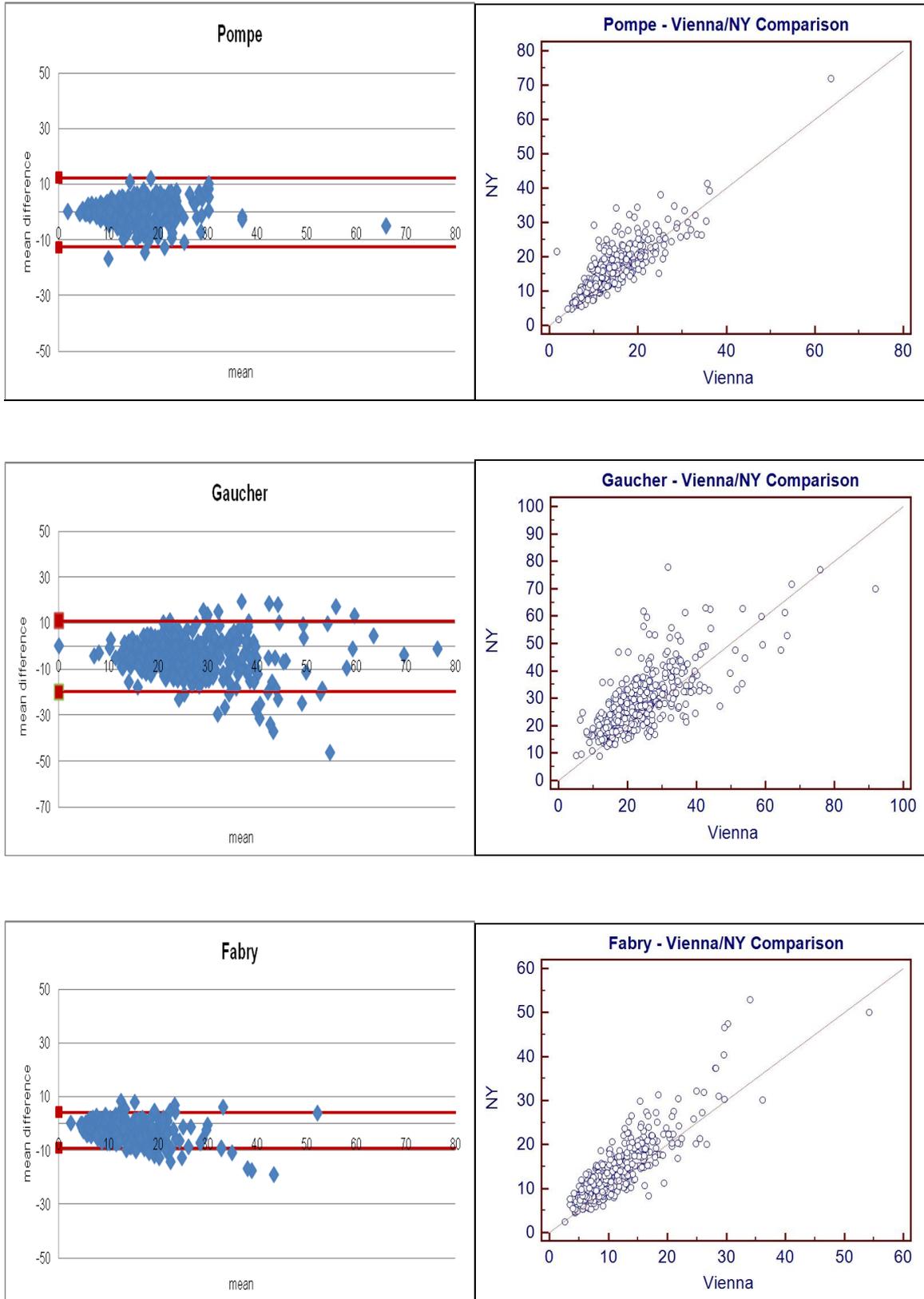


Fig. 7 Bland-Altman graphs and concordance correlation graphs for the multiplex assay comparison in the two laboratories

An acceptable correlation of results confirms the reliability of using DBS even for long distance sample transfer; no biological deterioration was observed in samples coming from NY and analyzed in Vienna, compared to the same samples analyzed in NY just after spotting.

Anyway, the correlation could be improved. Therefore, to establish if these differences were due to the assay rather than to the tandem mass analysis all the plates were dried and sent to the other laboratory to repeat the measurement.

A very good correlation related to the instrumental analysis was detected. On the other hand more variability related to the samples preparation, was reported, mainly for β -Glucosidase assay (Tab. 6, Fig. 8).

ABG	mean	st dev	upper limit	lower limit	ρ_c	ρ	Cb	Spread %
Total comp.	-4,5	7,8	10,9	-19,9	0,68	5,13	6,39	33,1
MS/MS comp.	-4,2	4	3,6	-12,2	0,85	0,93	0,91	21,7
Assay comp.	-0,3	7,9	15,2	-15,8	0,7	0,7	0,99	26,2

GAA	mean	st dev	upper limit	lower limit	ρ_c	ρ	Cb	Spread %
Total comp.	-0,1	6,33	12,2	-12,5	0,8	0,83	0,95	23,44
MS/MS comp.	-2,1	1,6	1,2	-5,4	0,95	0,99	0,96	15,07
Assay comp.	0,8	3,3	7,2	-5,8	0,86	0,87	0,99	15,11

GLA	mean	st dev	upper limit	lower limit	ρ_c	ρ	Cb	Spread %
Total comp.	-2,4	3,3	4,2	-9,1	0,8	8,87	0,91	30,62
MS/MS comp.	0,6	0,3	1,4	-0,1	0,99	0,99	0,99	5,74
Assay comp.	-1,7	3,1	4,49	-7,9	0,83	0,87	0,95	26,6

Tab.6 Concordance values between 2 laboratories for ABG, GAA and GLA activities

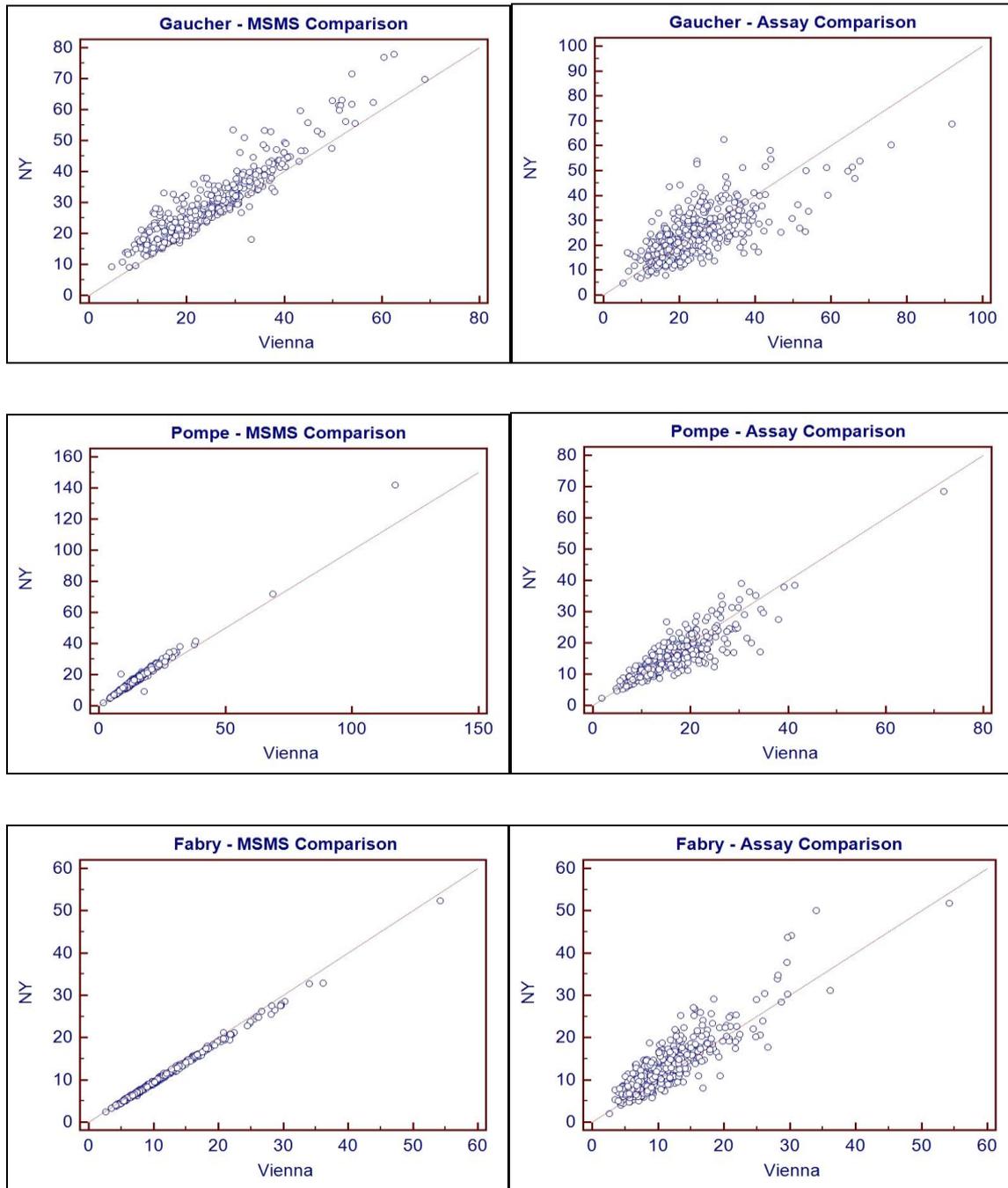


Fig. 8 MS/MS and assay concordance correlation for the ABG, GAA and GLA tests

For Gaucher assay no positives samples were detected. For Fabry assay only one sample presented low activity in both laboratories (Vienna 2.6 $\mu\text{mol/L/h}$; NY 2.4 $\mu\text{mol/L/h}$); instead, for Pompe assay one sample results positive both for Vienna 2.0 $\mu\text{mol/L/h}$ and NY 1.8 $\mu\text{mol/L/h}$, finally one showed low activity only in the analysis made in Vienna (1.6 $\mu\text{mol/L/h}$ against 21.5 $\mu\text{mol/L/h}$ in NY). Due to

the anonymous nature of the study it was not possible to repeat the analysis on samples detected as positive at first run.

Despite the use of different spectrometers and the work of different operators will inevitably lead to the introduction of biases, this work of comparison showed an acceptable correlation between laboratories: it showed that the tandem mass analysis is a robust and sensitive method for the metabolite analysis and that variability mainly depends on the procedure of samples preparation. For that reason the second part of my work was focused on the reduction of samples manipulation steps and on the optimisation of analytical parameters.

Moreover, the results obtained stressed the need of a strict coordination and of a protocol homogeneity between laboratories that want to be performed in collaboration these enzymatic assays.

3.3 Newborn screening pilot project

In 9966 anonymous newborn samples, obtained from the Szeged Hospital, β -Glucosidase, α -Glucosidase, α -Galactosidase and acid-Sphingomyelinase activities were measured.

Samples were first analyzed singly; provisional cutoff values have been used and respectively 4.0 $\mu\text{mol/L/h}$ for Gaucher, 2 $\mu\text{mol/L/h}$ for Niemann Pick A/B, 3 $\mu\text{mol/L/h}$ for Pompe and 2.5 for Fabry Disease.

At the first analysis 348 samples (2.23%) gave anomalous values (141 Gaucher, 42 Niemann Pick A/B, 49 Pompe and 116 Fabry), therefore they were retested in duplicate.

After a second analysis 41 samples (0.15%) were confirmed to have low enzyme activity (8 for Gaucher, 0.08%; 2 for Niemann Pick A/B, 0.02%; 15 for Pompe, 0.151% and 16 for Fabry, 0.161%) (Tab. 8).

Sample	Enzyme	Cutoff	1.Test	Retest 1	Retest 2	Result
6G3	ABG	4	3,2	3,4	3,7	carrier
14C5	ABG	4	3,4	3,0	2,8	carrier
17B8	ABG	4	3,6	3,2	2,9	carrier
19H7	ABG	4	3,7	1,9	2,2	carrier
14F10	ABG	4	3,8	3,8	3,3	carrier
20L2	ABG	4	3,9	2,8	3,2	normal
24F8	ABG	4	3,8	3,6	2,4	normal
26D4	ABG	4	3,5	2,4	2,7	normal
21C5	GLA	2,5	2,3	1,6	1,6	normal
8H2	GLA	2,5	2,4	1,9	2	normal
46H4	GLA	2,5	2,2	1,9	2	normal
54B8	GLA	2,5	2,4	2,6	2,5	normal
57I9	GLA	2,5	2,5	2,3	2,3	normal (SNPs)
58H9	GLA	2,5	2,3	2,2	1,9	normal
80D2	GLA	2,5	2,1	2,3	2,9	normal
70D7	GLA	2,5	2,5	1,9	1,9	normal (SNPs)
75I7	GLA	2,5	2,4	2,3	2,7	normal
83E1	GLA	2,5	1,8	2	1,5	normal
63G4	GLA	2,5	1,7	1,7	1,7	unclear
63E3	GLA	2,5	2	2,5	2,2	normal (SNPs)
60G8	GLA	2,5	1,8	1,9	2,2	normal
85C2	GLA	2,5	1,9	1,8	2	normal
85C9	GLA	2,5	2,1	2,1	1,8	unclear
63A7	GLA	2,5	2,1	2	2,4	normal (SNPs)
11i2	GAA	3	2,1	2,3	2	carrier
25F4	GAA	3	2,6	2,8	2	pathologic
39F2	GAA	3	3,1	2,4	2,6	normal
43B3	GAA	3	2	1,6	1,6	pathologic
43H7	GAA	3	1,6	1,5	1,7	carrier
46E10	GAA	3	2,8	2,5	2,5	carrier
47H7	GAA	3	2,7	2,6	2,9	carrier
75I4	GAA	3	3	2,6	2,6	carrier
76A4	GAA	3	2,9	2,4	2,3	normal
80C5	GAA	3	2,9	2,6	2,5	normal
80F9	GAA	3	1	0,7	0,8	carrier
80I4	GAA	3	2,6	1,9	2,6	normal
72G6	GAA	3	2,7	2,8	3	normal
85E1	GAA	3	3	1,7	1,9	normal
87D10	GAA	3	2,3	2,4	2,7	normal
55D7	ASM	2	0,9	1,6	1,9	normal
87C3	ASM	2	1,6	2,4	1,9	normal (SNPs)

Tab. 8 Molecular analysis result for samples that presented low enzymatic activity

These samples were sent to a third laboratory for the molecular analysis, that could isolate genomic DNA directly from the DBS card used for enzymatic tests.

Molecular analysis of the β -Glucosidase gene

Of the 8 samples requested for the β -Glucosidase analysis 3 resulted with no mutations; in five sample a C>T substitution in nucleotide 1223 was identified in heterozygosis. This gene variant was first described by Beutler and co-workers in 1996 in an European patient; it predicts a threonine-->methionine substitution in amino acid 369, however the associated severity is not known [105].

One patient presents a A>G transition in nucleotide 1226 in heterozygosis.

Previous studies have indicated that this A>G point mutation [106] is the most common genetic variant found in patients with Gaucher Disease type 1. It is responsible for the high incidence of GD in the Ashkenazi Jewish population [107,108].

Activity values of carrier samples varied from 1.9 to 3.8 $\mu\text{mol/L/h}$.

False positive rate for Gaucher Disease was 0.08%.

Molecular analysis of the α -Galactosidase gene

16 samples were submitted for alpha-galactosidase analysis: ten of them present no mutations, four present several polymorphisms, and were reported as negative. Finally two samples have an unclear result; in both samples the mutation c.937 G>T was reported in heterozygosis, resulting in an aspartic acid-to-tyrosine substitution (D313Y); in one sample 2 SNPs were detected (640-16A>G and 1000-22C>T).

The D313Y lesion was previously identified in classically affected males as the single mutation [109] or in cis with another missense mutation; Yasuda and co-workers demonstrated that the expressed D313Y enzyme (in COS-7 cells) was stable at lysosomal pH (pH 4.6), while at neutral pH (pH 7.4), it had decreased activity; D313Y is a rare exonic variant with about 60% of wild-type activity in vitro and reduced activity at neutral pH, resulting in low plasma alpha-Gal A activity [110]. These samples were reported as negative.

Activity of the two female samples with D313Y space from 1.7 to 2.1 $\mu\text{mol/L/h}$.

False positive rate for Fabry disease was 0.16%.

Molecular analysis of the α -Glucosidase gene

Seven of the 15 samples present no mutations; six samples carry non pathologic mutations in heterozygosis(4 with 664G>A, 1 with 1903 A>G, 1 with 841C>T).

In one sample the mutation 1552-3 C>G was identified in homozygosis; it is a splice-site mutations that cause the reduction of the number of correct splicing events by more than 90% and it is associated to a potentially mild phenotype [111].

One sample that reported two mutations in heterozygosis (1216 G>A, D406N and 1409 A>C, N470T) was classified as pathologic.

Heterozygote carriers shows an activity range of 0.7 to 3 $\mu\text{mol/L/h}$, that overlap to that of positive samples (1.6 to 2.8 $\mu\text{mol/L/h}$).

False positive rate for Pompe disease was 0.13%.

Molecular analysis of the acid-Sphingomyelinase gene

No mutations were detected in the two analyzed samples, in one of them 3 SNPs were detected; the false positive rate for NP disease was 0.02%.

Overall, the study identified 2 positives samples for Pompe Disease out of 9966 tested, one of them probably associated to a mild phenotype; for the other patient we had no information to predict the potential phenotype. This study also allowed the identification of carriers which were 5 for Gaucher Disease, 2 for Fabry Disease (one male and one female), and six for Pompe Disease, respectively. The false positive rate was 0.15%, meaning that 41 newborns would have to be recalled. This value is acceptable, although quite high, especially if considering the false positive rate obtained in the recently published LSD newborn screening in Austria (0.06%) [36]. Therefore, to render this value as low as possible it will be necessary to optimize some protocol steps.

From our study, the global incidence of these diseases, considering a population of 10000 samples, came out to be 1:4983, which is surprisingly high considering the currently accepted LSD frequency, but it is totally in line with the more recent works on LSD newborn screening [31,36].

Nowadays, in the Centogene Laboratory the screening program reached the 29000 samples with an incidence of 1:2416, including 7 confirmed Pompe patients, 2 Fabry and 3 Gaucher subjects (personal communication).

Also, newborn screening for Fabry Disease carried on in Taiwan identified a high frequency of the disorder (~1 in 1,250 males) with the majority of the identified patients(86%) carrying the later-onset phenotype (~1 in 1,390 males) [31]. Very recently Mechtler and co-workers published the first population screening study for Fabry, Gaucher and Niemann Pick A/B Diseases and they report a global incidence of 1:2315 [35].

In addition, several studies have reported potentially undiagnosed cases of Fabry Disease in specific populations as kidney transplant patients [112], patients with early-onset cryptogenic stroke or with hypertrophic cardiomyopathy [113,114].

All these results lead to the hypothesis that until now the LSD incidence has been underestimated

The comparison of the cut off values set in these different studies deserves some considerations. For Gaucher and Niemann Pick A/B Diseases the cutoff should be not lower than 4.2 $\mu\text{mol/L/h}$, and 2.5 $\mu\text{mol/L/h}$ respectively, that are the higher activity values of tested patients. For Pompe it is confirmed to be 3 $\mu\text{mol/L/h}$ since the higher activity in the confirmed positive samples was 2.8 $\mu\text{mol/L/h}$; and for Fabry 2.5 $\mu\text{mol/L/h}$.

Considering that each laboratory will define its own cutoffs values, depending on the specific arrangement of the protocol, only a wide screening project will allow a more precise and reliable definition of the cutoffs values, that could be varied a little, from the ones established in this work.

3.4 Assays set up in Padova

The five assay performed in Vienna were then reproduced in the laboratory in Padova.

Since in the first assays performed a high variability in the enzymatic activities analyzed was detected and taking also into consideration the results obtained with the previously mentioned comparison study, some protocol modifications were made. The aim was to reduce at a minimum the possibility to introduce

errors and biases in the pre-analytical phase, that results to be the most critical step due to a consistent samples manipulation.

The extraction and purification steps were eliminated and substituted with an Ultra Performance Liquid Chromatography analysis connected in line with the mass spectrometer. In this way the samples processing time was remarkably reduced, and above all, limiting the manipulation of samples, the probability to introduce biases, errors and deriving false positive results was drastically knocked down.

Solutions for liquid chromatography were optimized as follows:

A: H₂O + 0,05 % ACF

B: 50:50 ACN: MeOH + 0,1 ACF

Successively, since some substrate interference problems were detected for the peaks corresponding to ABG product and GALC product the UPLC gradient was modified to obtain a better peaks separation.

A higher water concentration was used at 1 minute time to obtain a better peaks package (Tab. 9).

Previous gradient					Optimized gradient				
Time (min)	Flow (ml/min)	Sol A (%)	Sol B (%)	Curve	Time (min)	Flow (ml/min)	Sol A (%)	Sol B (%)	Curve
0	0.3	50	50	6	0	0.3	50	50	6
1.5	0.3	2	98	6	1	0.3	25	75	6
4	0.3	2	98	6	2.5	0.3	2	98	6
4.01	0.3	50	50	6	4	0.3	2	98	6
5	0.3	50	50	6	4.01	0.3	50	50	6
					5	0.3	50	50	6

Tab. 9 Optimization of UPLC gradient

This modification was essential since, in these tests positive samples are detected for the reduction of the peak corresponding to the product of the enzyme activity; having an interference that could overlap with the peak of the analyte of interest, or that could make the spectra interpretation more complex, it will lead to false negative results.

To get an even better separation, high precision and sensitivity a perfusion column and a switch valve were introduced. As was reported in a recent paper by La Marca and co-workers [115] this additional online sample purification step guarantees the maximum sensibility and reliability of the analysis, so that all enzyme activities show an unambiguous difference between DBS from healthy controls and LDS affected patients.

Samples passing through the perfusion column are deprived of impurities, salts and interferers and this ameliorates the definition of the peaks.

The challenge is to reduce at a minimum the false positive rate that is still one of the main controversial points of discussion for the inclusion of LSDs in newborn screening programs because of the high level of stress for families with a re-called baby.

Solvents:

A: H₂O + 0.05 ACF

B: MeOH

Parameters:

Time (min)	Sol. A (%)	Solv. B (%)	Flow (µl/min)
0	98	2	400
0.01	98	2	800
0.5	/	100	800
0.51	/	100	800
2	/	100	800
2.01	/	100	800
3	/	100	800
3.01	98	2	800
4.99	98	2	800
5.5	98	2	400

Tab. 10 Elution parameters of the HPLC

MS/MS parameters were optimized as they are summarize in Tab.11

Analyte	Parent (Da)	Daughter (Da)	Dwell(s)	Cone (V)	Coll (EV)	Delay (S)	Ret time (min)
<i>IDUA-IS</i>	377,54	277,40	0,05	35	10	0,010	1,57
<i>IDUA-P</i>	391,45	291,20	0,03	35	10	0,010	1,60
<i>GLA-P</i>	484,57	384,40	0,05	35	15	0,010	2,07
<i>GLA-IS</i>	489,64	389,40	0,02	35	15	0,010	2,06
<i>GAA-P</i>	498,64	398,40	0,05	35	15	0,010	2,19
<i>GAA-IS</i>	503,61	403,40	0,02	35	15	0,010	2,18
<i>ASM-IS</i>	370,63	264,20	0,02	35	15	0,010	3,41
<i>ASM-P</i>	398,67	264,20	0,05	35	15	0,010	3,62
<i>GALC-P</i>	426,72	264,20	0,05	35	15	0,010	3,84
<i>GALC-IS</i>	454,82	264,20	0,05	35	15	0,010	4,07
<i>ABG-P</i>	482,80	264,20	0,05	35	15	0,010	4,38
<i>ABG-IS</i>	510,83	264,20	0,03	35	15	0,010	4,80

Tab. 11 MS/MS parameters for LSD analysis

Other MS/MS parameters are:

Capillary Voltage: 3kV

Source Temperature: 100°C

Desolvation Temperature: 200°C

Multiplier: 650V

Fig 9 shows the results of the assay set up and optimization: all the 12 peaks related to the metabolites under investigation are revealed with a good intensity, they are all well defined, with no problems of interference with other blood metabolites.

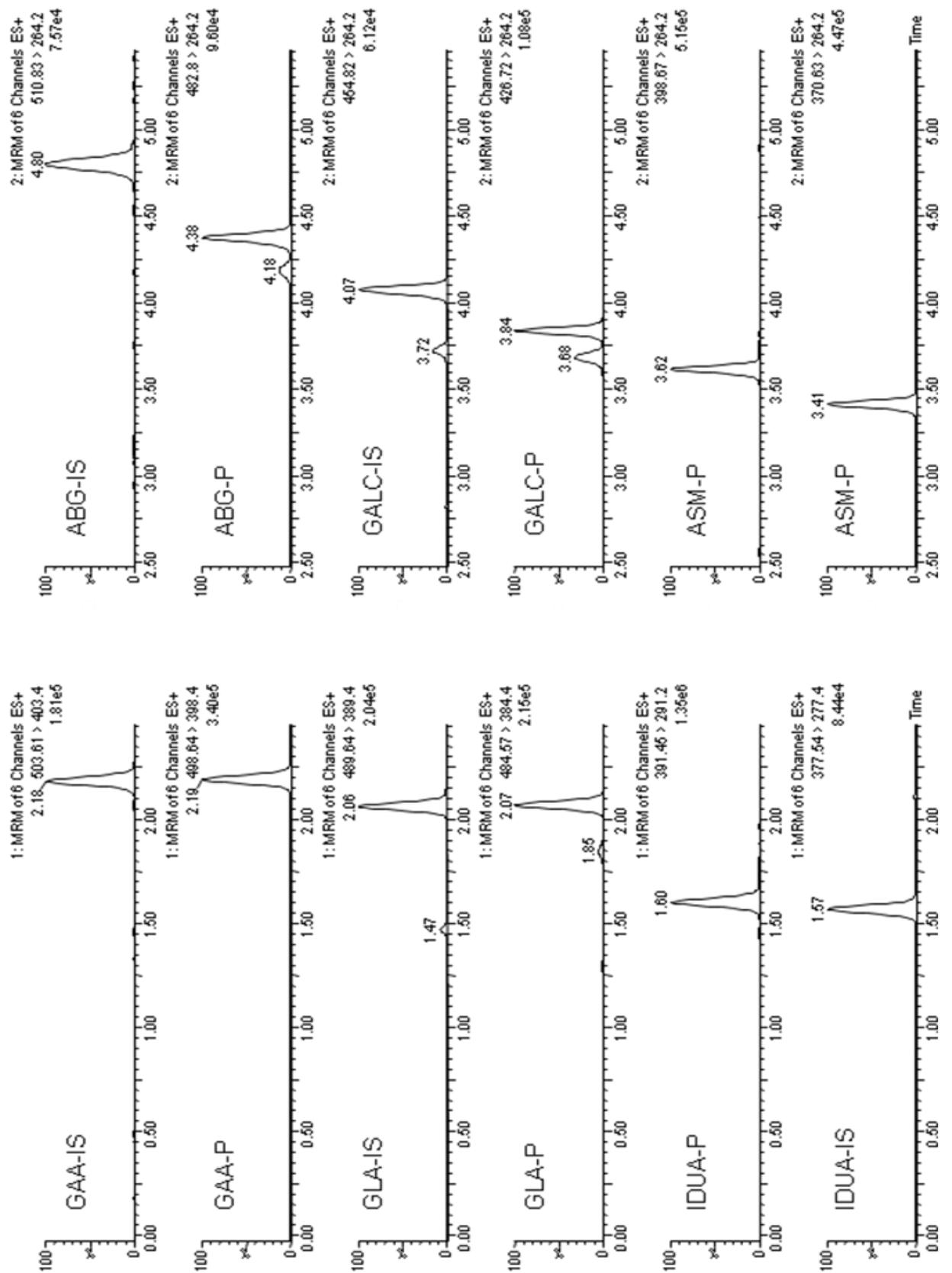


Fig. 9 The chromatogram shows the separation of the 12 peaks that correspond to the analytes of interest.

One confirmed positive Pompe and one confirmed positive Fabry sample were tested (Fig. 9,10).

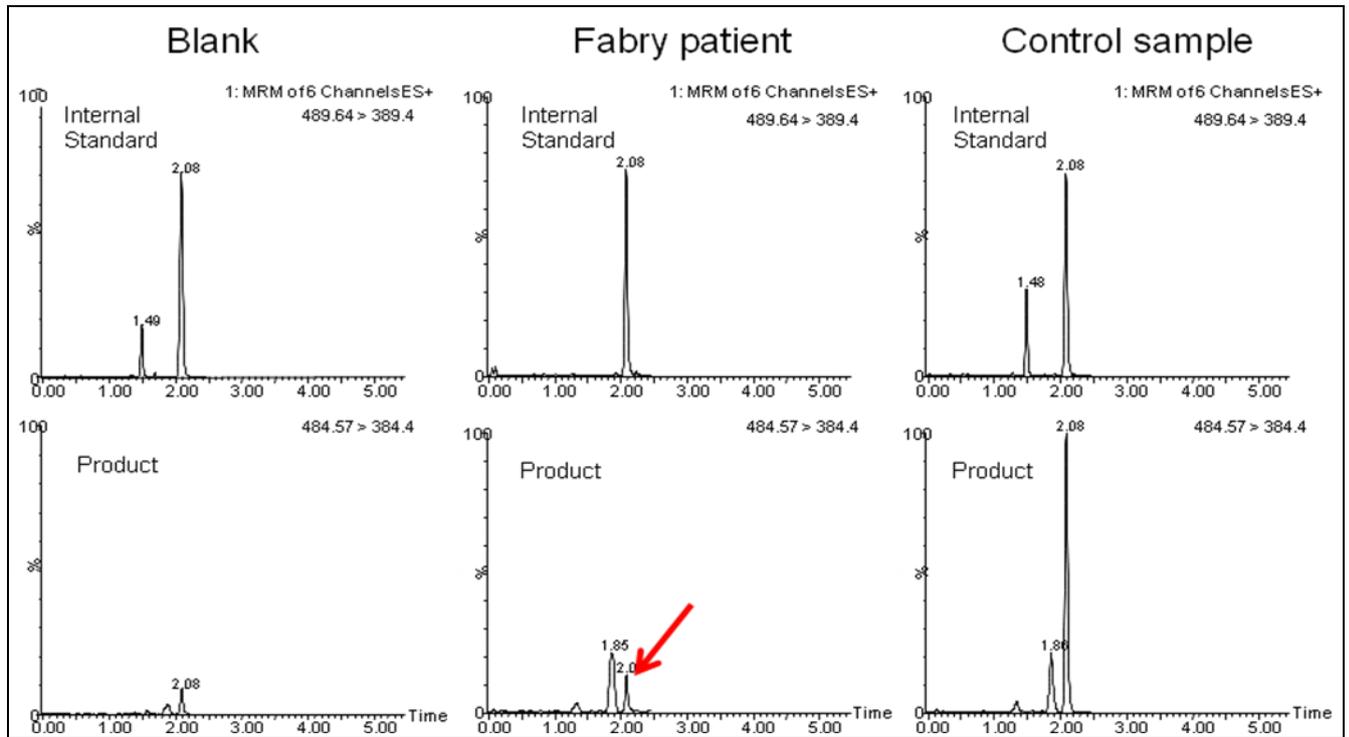


Fig.9 Chromatograms of a blank, a Fabry patient and an healthy control are reported: GLA Internal Standards peaks are shown in the upper part, while GLA product peaks lay in the lower part

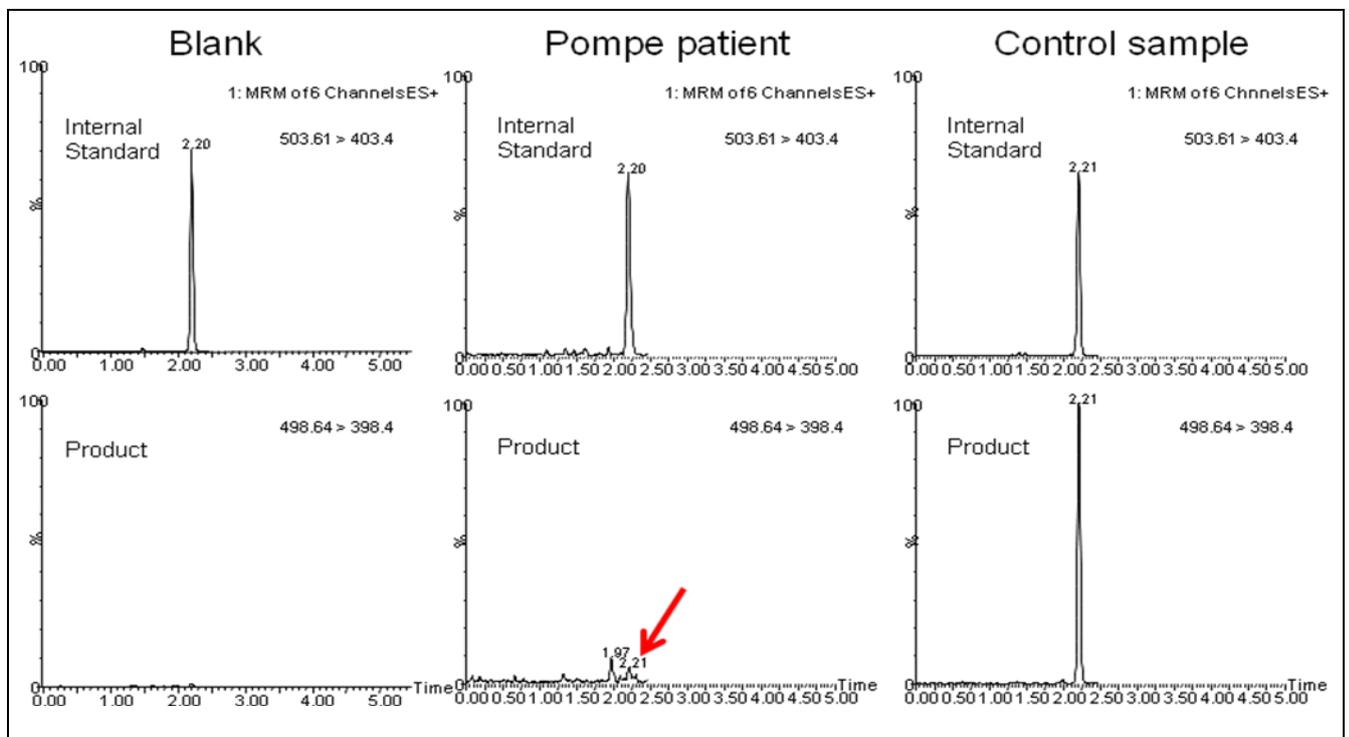
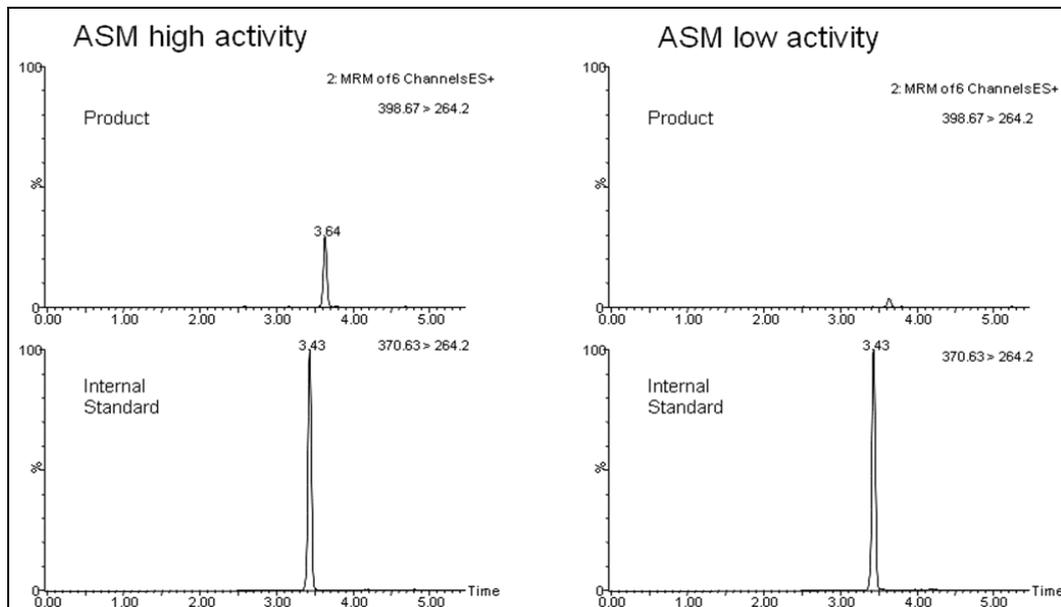
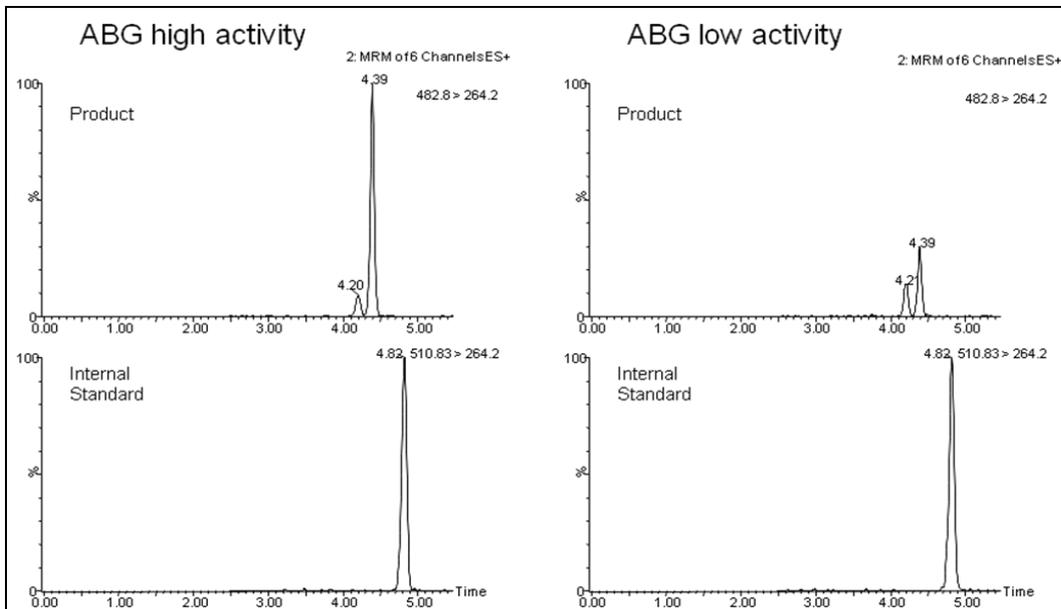
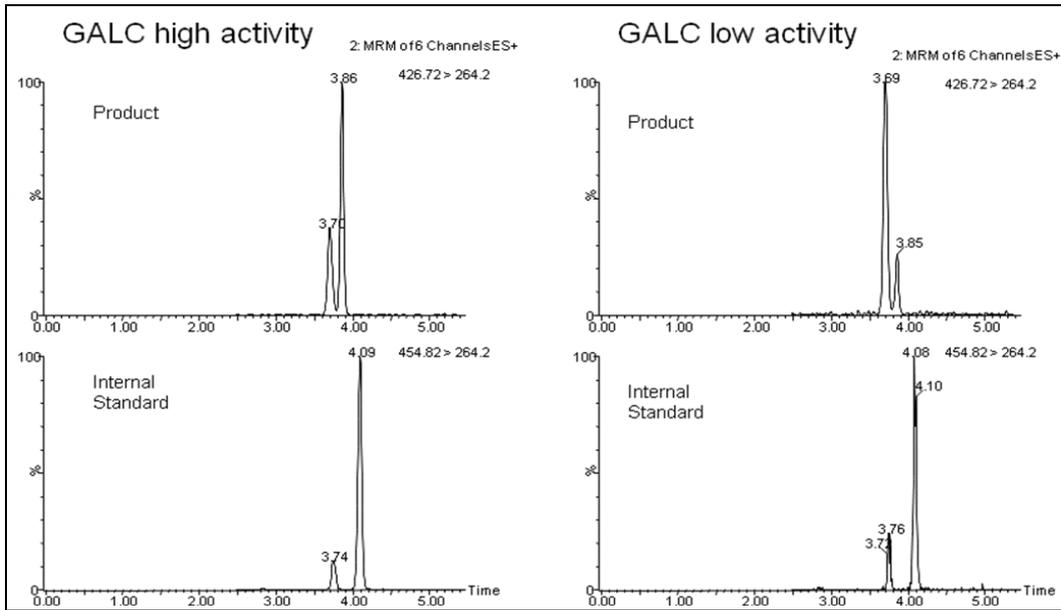


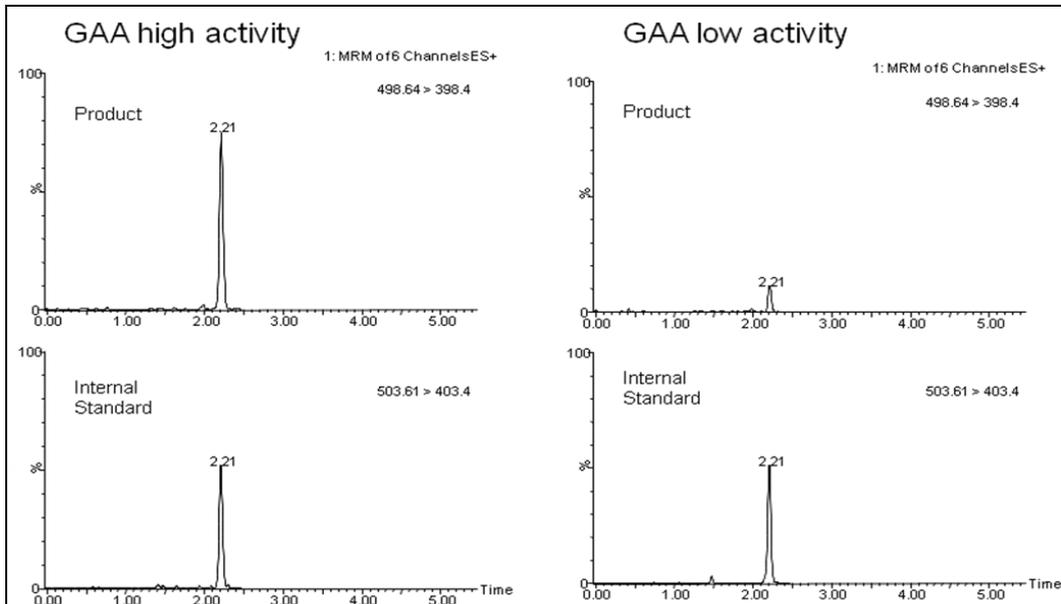
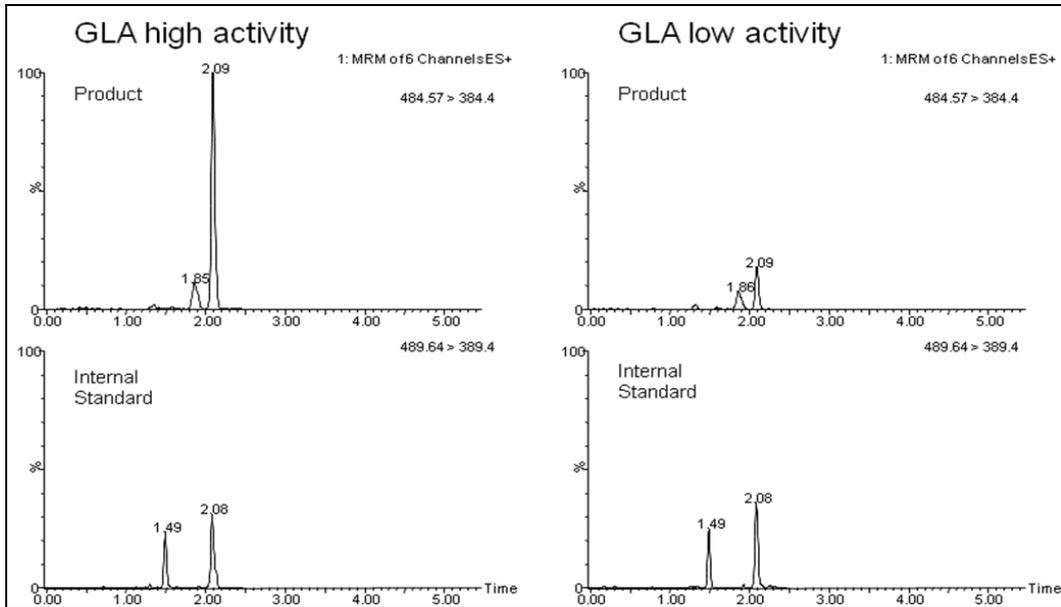
Fig.10 Chromatograms of a blank, a Pompe patient and an healthy control are reported: GAA Internal Standards peaks are shown in the upper part, while GAA product peaks lay in the lower part

As it is shown the Internal Standard peaks has the same intensity in the controls, in the blanks and in the pathological samples; instead the Product peak shows an unambiguous difference in the compared samples. In the positive samples the peak is significantly reduced: in Fabry DBS it is nearly 10% with respect to the Product peak in the control, and in Pompe DBS it is comparable to the ground noise.

Unfortunately, confirmed positive samples of other Disease were not available, therefore an evaluation of the assay performance was obtained using CDC quality controls (low, medium and high activities) (Fig.1)







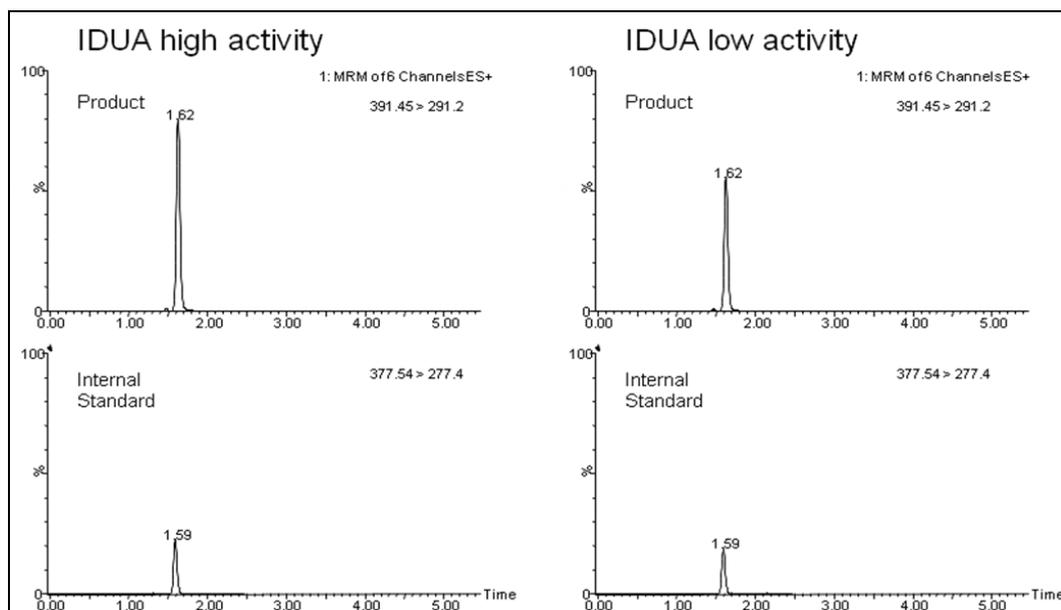


Fig. 11 Chromatograms of low activity and high activity samples are shown for the six enzymatic tests

The reduction of the samples manipulation during the pre-analytical phase combined with the introduction of the perfusion column and the UPLC, whose analytic parameters were minutely optimized, has led to an excellent peaks isolation. Impurities were mainly removed thanks to the perfusion column, allowing to avoid interference problems in samples ionization and detection and to maximize sensitivity and precision.

Moreover, in order to avoid to misinterpret as “positive” a sample in which the assay failed for any experimental reasons, it is mandatory that each plate includes some control samples (positive and negative), although the performance of a multiplex assay contains in itself an internal control: in fact, the detection of a low enzyme activity for more than one enzyme is a sign of sample degradation or of a problem occurred during the enzyme reaction and not of a pathological condition.

Then, since results are expressed starting from a ratio between the generated product and the internal standard (undergoing the same process of samples), no false positive results due to the loss of sample along the preparation steps will be produced.

4. CONCLUSION

Lysosomal storage disorders (LSDs) are a wide and complex group of inherited progressive diseases.

In the last decade, the research on LSDs has led to remarkable advancements in terms of innovative therapeutic approaches and of new diagnostic methods.

For the most diffused of these pathologies recombinant functional enzymes have been developed for the so called Enzyme Replacement Therapy (ERT) which, together with the hematopoietic stem cell transplantation, is the main form of treatment for LSDs. It is nowadays evident that these therapeutic strategies would need an early application to maximize their benefits. It is, in fact, of primary importance that treatments start before the development of irreversible damages.

On the other hand, recently the application of tandem mass spectrometry analysis has allowed the set up of a safe, rapid, economic, non-invasive and highly reliable method for the detection of enzymatic activities in Dried Blood Spots (DBS). For ethical reasons, the diseases taken into consideration were primarily those that can already benefit of a therapy.

This study confirms the stability of lysosomal enzymes in DBS for months if stored at 4°C or lower temperatures; moreover, with DBS a delivery of the samples at room temperature with no problems of deterioration is feasible, thus favouring the creation of a few centralized reference laboratories for their analysis.

Precision and reproducibility of tests even between different laboratories were demonstrated in this study, enabling the application of the method to different purposes.

First of all it can be used to rapidly evaluate the enzyme bioavailability in single individuals.

Since, until now, ERT protocol is standard for all patients (weekly or biweekly infusions as mg/kg) [116], and since still very little is known on the individual determinants of therapeutic efficacy, the application of this method to monitor the enzyme up-take in patients undergoing ERT will facilitate the development of personalized therapeutic protocols, thus reducing unnecessary treatments and side effects and leading to a more cost-effective healthcare.

Enzymatic tests could even be proposed for defined high risk populations presenting specific symptoms, as it has been reported in several recent studies on kidney transplant patients, patients with early-onset cryptogenic stroke or with hypertrophic cardiomyopathy, that identified previously unrecognized individuals with Fabry disease [112,113,114].

Finally, the feasibility of a multiplex assay, allowing the simultaneous analysis of several enzymes for a high number of samples, represents a necessary condition to allow LSDs to be included in screening projects.

As was done here for MPS I, it is also possible to implement the multi-test with assays for other LSDs, as soon as therapies and reagents will be available for them.

In fact, considering that the general criteria of the World Health Organization to add a disease in the screening panel are, mainly, the relatively high frequency, the availability of a treatment and of a reliable test suitable for high throughput analysis and a lower screening/therapies cost with respect to the care of untreated patient, LSDs could be a good candidate to be added to the in force screening programs [98].

My experience in the pilot screening work on Hungarian samples demonstrates as a project of screening for LSDs is not only feasible, but, with DBS and tandem mass analysis is also rapid, reliable and easy to organize.

Moreover the protocol optimization conducted in Padova has reduced at a minimum the possibility to introduce operator's mistakes and has rendered the analysis even more sensitive and precise, significantly reducing the risk to obtain false positive values.

The use of MSMS will offer more internal controls compared to fluorometric test, in fact analysing several enzyme simultaneously will permit to discriminate if a positive result is due to a real enzyme deficiency or simply to samples deterioration (if more than one enzyme present low activity).

Besides, having an internal standard for each enzyme reaction, that undergoes to the same process of samples, will guarantee an easier critical interpretation of positive results.

Since the false positive problem is one of the most controversial point in the decision to include or not LSDs in newborn screening programs, as reported by

Ross [117], we believe that this optimized protocol could be a challenge in this direction.

On the opposite, there are no doubts that a newborn screening program will be essential for a precocious identification of true positive samples, and that a prompt therapeutic intervention in early onset patients will maximize its efficacy and will also guarantee benefits in terms of patients' lifestyle and compliance, families management and sanitary costs.

The time of screening for the late-onset variants, especially for Fabry disease, and the time to start the therapy in non-symptomatic patients still need to be critically discussed, since opposite positions are debating and particularly strong is the position of who discourages the testing of minors for adult onset conditions. The aim of clinicians is now to overcome this problem: guidelines for diagnostic confirmation and clinical management of presymptomatic individuals suspected to have a lysosomal storage disease were recently published [118].

Interestingly, the Tay-Sachs disease (TSD) carrier screening, initiated in the '70s, has reduced the birth-rate of Ashkenazi Jews with TSD worldwide by 90%.[119].

Moreover, the positive results in term of clinical outcome in screened populations (as it was reported for Pompe [30,120] and Krabbe [121]), or in precocious detected patients [122] strongly support the screening direction; this tendency was recently confirmed with the insertion of LSDs in the screening panel of the State of New Jersey.

http://www.huntershope.org/site/PageServer?pagename=newsmedia_landing&autologin=true).

Another relevant question in debating is whether the test should be applied to the most common disorders or if it should be limited to pathologies for which an early diagnosis would allow early treatment: Hayes and colleagues assessed the opinions of parents of MPS patients regarding the possibility of newborn screening for this condition [123]. Most of them would support it even when diagnosis would not lead to the application of a therapeutic protocol.

In addition, a screening program will permit to reveal the real incidence of these pathologies, too often underestimated, with an important fallout both on the attention of the medical community to LSDs, and on the future plans of National Health Care Systems with respect to these disorders.

An early detection of pathologic mutation will be the basis for genetic counseling on reproductive risk or prenatal diagnosis.

Acknowledgements

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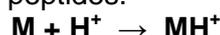
Appendix A

A more detailed description of mass spectrometry characteristics

Sample Ionization

According on samples nature and on witch pattern of information you are interested in, several ionization techniques could be used; they include protonation, deprotonation, electron ejection, electron capture, cationization, or by transferring a charged molecule from a condensed phase to the gas phase.

- **Protonation:** a proton is added to on the more basic residues of the molecule, such as amines, to form stable cations, and producing a net positive charge of +1 for every proton added. It is very used for peptides.



Advantages: many compounds could accept H^+ ; many ionization sources, as ESI, APCI, FAB, CI and MALDI will generate these species.

Disadvantages: some compounds result not stable to protonation or cannot accept a proton easily (ex. Carbohydrates or hydrocarbons respectively).

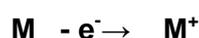
- **Deprotonation:** it is obtained by the removal of a proton from a molecule, giving the net negative charge of -1.



Advantages: It is very useful for acidic species including phenols, carboxylic acids and sulfonic acids; it is commonly achieved via MALDI, ESI, FAB or APCI.

Disadvantages: compound specific

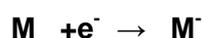
- **Electron ejection:** a 1^+ net positive charge it is obtained by the ejection of an electron. It is used with relatively non-polar compounds with low molecular weight.



Advantages: associated with electron ionization (EI); it provides molecular mass as well as fragmentation information.

Disadvantages: it could generate too much fragmentation, it could be difficult to establish whether the highest mass ion is the molecular mass or a fragment.

- **Electron capture:** a net negative charge of 1- is achieved with capture or absorption of an electron. It is a recommended method for molecules with high electron affinity, as halogenated compounds.

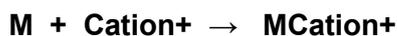


Advantages: associated with electron ionization (EI); it provides molecular mass as well as fragmentation information.

Disadvantages: it could generate too much fragmentation, it could be difficult to establish whether the highest mass ion is the molecular mass or a fragment.

- **Cationization:** a positively charged ion (other than a proton, ex. alkali, ammonium) is added to a neutral molecule to form a non-covalent charged complex. Since the

charge remains localized on the cation, the fragmentation is minimized. Carbohydrates are excellent candidates for this ionization mechanism with Na⁺.



Advantages: many compounds will accept a cation, such as Na⁺ or K⁺; many ionization sources could generate these species: ESI, APCI, FAB and MALDI.

Disadvantages: cationized molecules give limited fragmentation information in tandem mass spectrometry experiments.

- **Transfer of a charged molecule to the gas phase:** a compound already charged in solution is transferred through the desorption or ejection of a charged species from the condensed phase into the gas phase.



Advantages: useful when the samples is already charged; many ionization sources could generate these species: ESI, APCI, FAB and MALDI.

Disadvantages: only for pre-charged ions.

Ionization sources

- **Electrospray Ionization (ESI)**

The first electrospray experiments date back to the 1930s (Chapman), but it's only in the late 60s that Dole coupled electrospray ionization and mass analysis, and discovered the phenomenon of multiple charging of molecules. The ESI modern technique and its application to biological macromolecules warranted Fenn the Nobel prize in 2002.

ESI is a soft desorption ionization method routinely used with fairly large mass molecules, as peptides, proteins, carbohydrates, small oligonucleotides ext.

It produces gaseous ionized molecules from liquid solution, at atmospheric pressure, by mixing the sample with a polar solvent, and pumping it through a stainless steel capillary which carries between 10⁻⁶ and 10⁻⁷ mV. The needle to which the potential is applied serves to disperse the solution into a fine spray of charged droplets ("Taylor cone") that undergo electrophoretic movement in response to the imposed electric field.

In the positive ion mode, anions migrate in the direction of the capillary, while cations migrate in the direction of the counter-electrode (vice versa for the negative ion mode).

Dry gas or heat, or both could be applied to the droplets at atmospheric pressure causing the solvent evaporation, the consequent reduction in droplet size and the increase of charge density. When the electrostatic repulsion becomes equal to the surface tension force, the "Rayleigh stability limit", droplets undergo to the "Coulomb fission", a process that produce smaller droplets. This event may occur several times, until small droplets contain one or more charge but only a single analyte molecule. It is called "charged residue model" and it was the first mechanism initially proposed by Dole and co-workers.

Another mechanism, the "ion evaporation mechanism" was proposed later by Iribarne and Thomson. They hypothesized, that a certain point before the Rayleigh stability limit, the electric field on the surface of the droplets is sufficient high that solvated ions may be emitted into the gas phase.

It is possible that the two mechanisms may actually work in concert: the charge residue mechanism dominant for masses higher than 3000 Da while ion evaporation dominant for lower masses.

In either cases emerging ions are directed into an orifice through electrostatic lenses leading to the vacuum of the mass analyzer. Since ESI involves the continuous introduction of solution, it is suitable for using as an interface with HPLC or capillary electrophoresis.

Advantages: practical mass range up to 70 000 Da, good sensitivity (high femtomole to low picomole), adaptable to liquid chromatography and to tandem mass analysis, multiple charging allows for analysis of high mass ions with a relatively low m/z instrument, no matrix interference, no sample degradation.

Disadvantages: carryover from sample to sample, essential sample purity, not ideal for complex mixtures.

- **Nano Electrospray ionization (nano-ESI)**

It was originally described by Wilm and Mann (1996), and it utilized the same ESI fundamental ionization process.

The main differences between conventional electrospray and nano-ESI is its 1-2 μm spraying orifice, its very low flow rate of $\sim 20\text{nL}/\text{min}$ (nowadays even few nL/min), the smaller size of droplets it generates and the absence of solvent pumps and inlet valves.

It is typically used for peptides and proteins and strongly recommended for impure samples, as peptide mixtures.

Advantages: mass range up to 70 000 Da, few μl of samples needed, less interference effects of salts or other species, high sensitivity (high zeptomole to low femtomole), very useful for impure mixtures, freedom to choose solvents in a wide range of composition and pH, no sample degradation.

- **Atmospheric pressure chemical ionization (APCI)**

Although ESI and APCI were published in parallel, APCI was not widely used until the late 80s.

The sample solution is introduced directly in the ionization source, that contains an heater vaporizer which allows a rapid vaporization of the droplets. Vaporized molecules are then carried through an ion-molecule reacting region at atmospheric pressure.

Here a corona discharge produces primary ions, N_2^+ , N_4^+ by electron ionization; the collision of these ions with the vaporized solvent molecules produces secondary reactant gas ion as H_3O^+ , or $(\text{H}_2\text{O})_n\text{H}^+$ that undergo to repeated collision with the analyte resulting in the formation of the analyte ions.

Proton transfer, MH^+ reactions or electron transfer or proton loss, $[\text{M}-\text{H}]^-$, occurs in the positive or in the negative mode. Finally ions enter in the pumping and focusing stage.

Advantages: mass range up to 1200 Da, combination with HPLC or other separation techniques, spectra of predominantly molecular species and adduct ions with very little fragmentation, useful for very large and neutral molecules as lipids.

Disadvantages: not for thermally labile compounds, no multiple charging, lower sensitivity than ESI (high femtomole).

- **Matrix-assisted laser desorption/ionization (MALDI)**

It was first introduced, in 1988, by Hillenkamp and Kavas: it is a solid phase techniques that uses a laser energy for a non destructive vaporization and ionization of both large and small biomolecules.

The analyte is co-crystallize with a matrix compound in large molar excess, usually a UV-absorbing weak organic acid; a laser irradiation of this mixture results in the vaporization of the matrix, which carries the analyte with it.

The matrix has a double function, on one hand it plays a key role by strongly absorbing the laser light energy and causing indirectly the analyte vaporization. On the other hand it serves as proton donor and receptor providing the analyte ionization both in positive and negative modes. Two main theories have been proposed for desorption of large molecules by MALDI: the "thermal spike model" and the "pressure pulse theory". The first assumes that the matrix molecules sublime from the surface due to a local laser heating at low fluence; poor vibrational coupling between matrix and analyte lead to a low fragmentation level. The pressure pulse theory provides that large molecules are desorbed by a momentum transfer from collision with matrix molecules, thanks to a pressure gradient from the matrix normal to the surface. In any case ionization (generally protonation or cationization) depends critically on matrix-analyte interactions.

Advantages: good for analysis of complex biological samples and for synthetic polymers which have low volatility, low to high femtomole sensitivity, good for masses bigger than 200000 Da (up to 300000 Da), little to no fragmentation is possible, tolerance of salts in mill molar concentration

Disadvantages: photo-degradation by laser desorption/ionization can occur, not recommended for small molecular weight compounds, because matrix related ions could overlap with mass range of interest, degradation of some compounds if acidic matrix is used, photo degradation and matrix reactions.

- **Desorption/ionization on silicon (DIOS)**

It was introduced in 1999 by Siuzdak and co-workers which used a laser irradiation to vaporize and ionize the analytes trapped in a porous silicon. The structured silicon geometry provides a scaffold for retaining solvent and analyte molecules; furthermore the high area surface and the UV absorption properties of the silicon are crucial for the desorption/ionization process. It is also possible to perform different chemical or structural surface modifications to optimize the process.

The DIOS technique produces the same low fragmentation level as MALDI, but it supplies, thanks to no matrix interference, a good sensitivity for small molecules.

Advantages: mass range up to 3000 Da, little or no fragmentation, no matrix dependence, no interference and consequently low background in the lower mass range, good for complex mixtures, low femtomole to high yoctomole sensitivity.

Disadvantages: rapid molecular degradation observed upon direct exposition to laser irradiation

- **Fast atom/ion bombardment (FAB)**

Similar to MALDI, FAB provides a matrix and a highly energetic beam of particles to desorb ions from a surface.

FAB technology uses a non-volatile liquid matrix to constantly supply new sample to the surface as it is bombarded by the incident ion beam. Matrix, commonly n-nitrobenzyl alcohol and glycerol, also prevents the analyte from degradation by adsorbing most of the incident energy.

Unlike MALDI, FAB uses a continuous ion beam of Xe neutral atoms or Cs⁺ ions to sputter sample and matrix in the gas phase; the analytes could be charged before desorption or they may become charged during FAB through reactions with surrounding molecules or ions.

In FAB spectrum matrix ions and protonated/cationized molecular ions of analyte are detected.

Advantages: mass range up to 7000 Da, good for non-volatile compounds, few fragmentation

Disadvantages: 1000 less sensitive than MALDI, samples should be pure and free of additional salts, buffer etc., matrix interference, matrix reaction and some photo degradation are possible, very limited for complex mixtures, nanomole sensitivity.

- **Electron ionization (EI)**

In this hard ionization source for volatile compounds, where the sample is vaporized before be passed through a continuous electron beam, that is generated by heating a filament. The ejected electrons are accelerated with an electric field at 70eV and, impacting with gaseous molecules, they transfer part of their kinetic energy to the analyte. This transfer results in analyte ionization, and the excess of energy taken over by the analytes (not more than 6 eV) leads to some degree of fragmentation. Adoption of this 70 eV standard has allowed to create libraries to identify molecules by their unique fragmentation pattern.

It is possible to work both in positive and negative mode, even if electron capture is usually much less efficient than electron ejection, but it is recommended for high electron affinity samples.

An EI source is often coupled with a gas chromatography capillary that provides analytes separation.

Advantages: high fragmentation, no matrix interference, picomole sensitivity

Disadvantages: limited mass range (up to 500 Da) due to thermal desorption requirement, only for thermally stable molecules, not so useful with complex mixtures, unless used with GC

- **Chemical ionization (CI)**

Ideal for volatile compound that do not give molecular ions in EI. In this technology a reagent gas (Methane, isobutene, ammonia) is used to transfer proton to the sample, producing $(M+H)^+$ ions, that have not much tendency to fragment, being even electron species and little excess energy is imported to them. The reagent gas is ionized by an electron beam, and the resulting ions, at high pressure, undergo a complex series of ion-molecules reaction to produces charged species that finally collide with the analyte that gives or takes a proton.

For analytes that have electron-capturing moieties (ex. fluorine atoms or nitrobenzyl groups), negative chemical ionization is used, with the limit that molecules are often chemically modified with an electron capturing moiety prior analysis.

Advantages: Ion fragmentation determines the number of labile hydrogenous, it is possible to selectively ionize ammine, limited but powerful approach for certain derivatized molecules such as steroids, no matrix interference, picomole sensitivity.

Disadvantages: limited mass range (up to 500 Da), only for volatile and thermally stable compounds, not so useful with complex mixtures, unless used with GC.

Mass analyzer

- **Magnetic sector**

A magnetic sector alone will separate ions according their m/z . An electric field is used to accelerate ions into a magnetic field, perpendicular to the direction of ion motion, where they assume a circular trajectory with a radius that depends on the speed of the ion, the magnetic field strength and the ion m/z .

However, since ions leaving the ion source do not all have exactly the same energy and therefore do not have exactly the same velocity, the resolution will be quite low. In order to improve this, it is necessary to add an electric sector, with a force perpendicular to the direction of ion motion as well, to focuses ions according to their kinetic energy.

The most common way to use this double sector instrument is keeping the accelerating potential and the electric sector at a constant potential and to varying the magnetic field. Ions that have a constant kinetic energy, but different mass-to-charge ratio are brought into focus at the detector slit.

An alternative is to hold the magnetic field constant and scan the electric potential. This has the advantage that the electric field is not subject to hysteresis, so the relationship between mass-to-charge ratio and accelerating voltage is a simple linear relationship. These double-focusing mass analyzers are used with ESI, FAB and EI ionization, but owing to their large size and the success of time-of-flight, and of the quadrupole and Fourier Transform Mass Spectrometry (FTMS) analyzers with ESI and MALDI, they are not widely used.

Characteristics

Accuracy: < 5ppm

Resolution: 30 000

m/z range: 10 000

Scan speed: ~a second

Tandem mass: MS MS, but with limited resolution, high collision energy

It is the “classical” model against which other mass analyzers are compared, very high reproducibility, best quantitative performance of all mass spectrometer analyzers, high sensitivity and high dynamic range.

Disadvantages: Not well-suited for pulsed ionization methods as MALDI, usually larger and higher cost than other mass analyzers.

• **Quadrupole**

The most common mass analyzer in existence today is the quadrupole, used with EI source since the 1950s, and then coupled with ESI and APCI. It is made up of 4 axial rods through which ions pass on their way to the detector.

Two rods, positive and negative, are coupled two by two, and they are connected to a radio frequency (RF) generator and a DC potential.

What's happened to ions when they pass through the quadrupole, in positive ion scan?

- First electrodes pair (with a positive DC): since RF has a positive and a negative phase, during the first one ions are pressed in the centre between bars, instead during the negative phase ions tend to deviate from the centre according to the electrodes distance. Lightest ions will oscillate more and they will be lost.
- Second electrodes couple, (with a negative DC): during the negative phase ions stay close to the negative electrodes, while during the positive phase ions are pressed in the centre. In this case are heaviest ions that will be lost.

A logical consequence is that varying DC potential (U) and the applied RF potential (V(t)) and the RF frequency (ω) is possible to select which m/z ions are allowed to reach the detector.

Considering two parameters **a** and **q**, is possible to identify the region of ion stability, being:

$$a: \frac{4eU}{mr_0^2\Omega^2}$$

$$q: \frac{2eV}{mr_0^2\Omega^2}$$

The area at the apex of the stable region identifies the ions that will reach the detector. It is possible to vary U and V maintaining $a/q=(2U/V)$ constant and consequently changing the slope of the scan line: decreasing the slope means to decrease the resolution because more ions will be detected and vice versa.

Characteristics

Accuracy: 100ppm

Resolution: 4 000

m/z range: 4 000 (very useful for proteins and biomolecules ionized by EI that produce charge distribution from m/z 1000 to 3500)

Scan speed: ~a second

Tandem mass: Triple quadrupole, good accuracy and good resolution, low-energy collisions.

It tolerates quite high pressure, it is a relatively low cost instrument with a good reproducibility and it is easy to switch pos/neg ions.

Disadvantages: limited resolution, peak heights variable as a function of mass (mass discrimination), not recommended for pulsed ionization methods.

● **Ion traps**

Quadrupole ion trap or Paul trap

Three different subtypes have been developed depending on space configuration: the three-dimensional trap (quadrupole ion trap QIT), the linear trap (Linear trap quadrupole LTQ) and the cylindrical ion trap.

Generally an ion trap is made by three hyperbolic electrodes: a central ring, an entrance and an exit end caps electrodes that form a cavity in which ions are trapped.

A DC potential is applied to the two end caps, instead an RF potential of constant frequency but variable amplitude is applied to the ring electrode to produce a three-dimensional potential field within the trap.

Ions are created by EI, ESI or MALDI and are focused and pumped into the ion trap by an electrostatic ion gate that pulses open (-V) and closed (+V), once inside the trap they start a stable oscillating trajectory.

Usually ion traps are filled with helium (1 mtorr) that, increasing molecule collisions, lead to decrease ions kinetic energy and to focus ions in the centre of the trap.

The following formula describes ion motion according some parameters: the trap size (r), the oscillating frequency of the fundamental RF (w) and the amplitude of the voltage of the ring electrode (V).

$$qz = 4eV/mr^2w^2$$

To eject ions two ways are possible: the "Mass-selective axial instability mode" or the "Resonant Ejection mode".

In the first case a mass spectrum is generated by sequentially ejecting ions from low m/z to high m/z by varying amplitudes of the fundamental RF potential at the ring electrode,

that sequentially make ion trajectories unstable. Ions are ejected through a hole in the end cap and detected using an electron multiplier.

In the second mode beside the RF potential at the ring electrode, an additional oscillating RF (AC) is applied at the end caps, so that ions are excited by resonance with the AC potential. The motion of ions with the same m/z , influenced by the AC, becomes bigger, and they are ejected together.

The very nature of trapping and ejection makes a quadrupolar ion trap especially suited to performing MS_n experiments in structural elucidation studies. It is possible to selectively isolate a particular m/z in the trap by ejecting all the other ions from the trap. Fragmentation of this isolated precursor ion can then be induced by CID experiments. The isolation and fragmentation steps can be repeated a number of times and is only limited by the trapping efficiency of the instrument.

➤ **Static trap or Penning trap**

A Penning trap is structurally very similar to Paul trap, but it uses a combination of a strong homogeneous axial magnetic field to confine particles radially and a quadrupole electric field to confine the particles axially. The motion of the ions in the resulting total potential is now a complex orbit composed of three independent harmonic motions: the epitrochoid.

The sum of these two frequencies is the cyclotron frequency, which depends only on the m/z and on the strength of the magnetic field. This frequency can be measured very accurately and can be used to measure the masses of charged particles.

Advantages: comparing to a Paul trap it has many advantages: there is no micro-motion and resultant heating of the ion due to dynamic fields, it can be made larger maintaining strong trapping, and moreover Penning-trapped particles can be cooled to an extent that renders second-order Doppler shifts negligible.

Characteristics

Accuracy: 100ppm

Resolution: 4 000

m/z range: 4 000

Scan speed: ~a second

Tandem mass: MSⁿ, good accuracy, good resolution and low energy collision.

It is easy to switch pos/neg ions.

➤ **Fourier transform Ion Cyclotron Resonance (FT-ICR)**

It was first published in the middle 1950s but it remained a largely academic tool until the application of FT methods in the early 1970s. The ion trap has a cubic shape with six electrodes faces coupled two by two, and it is located inside a high magnetic field (4,7 to 13 tesla), with a pressure in the range of 10^{-10} - 10^{-11} mBar, and a temperature closes to the absolute zero.

Ions that enter in that cell acquire a circular motion, whose frequency is dependent on their m/z ratio, but at this stage no signal is detected since the radius of their motion is too small.

It is then possible to apply a swept RF pulse across the excitation plates of the cell, so that each individual excitation frequency will couple with the ion natural motion.

The "resonant" ions will absorb energy and the size of their orbit will increase until they induce an alternating current between the detector plates. The frequency of this current is the same as the cyclotron frequency of the ions and the intensity is proportional to the number of ions.

When the RF gets out of resonance for a particular m/z the ions go back to their natural orbit, and the next m/z packet is excited. The rapid RF sweep applied will produce an image current that contain frequency components from all of the mass-to-charge ratios. The various frequencies and their relative abundances can be extracted mathematically by using a *Fourier transform* which converts a time-domain signal (the image currents) to a frequency-domain spectrum (the mass spectrum).

Characteristics

Accuracy: <5 ppm

Resolution: 100 000

m/z range: 10 000

Scan speed: ~a second

Tandem mass: MS^n , excellent accuracy and resolution of product ions.

It can be coupled with MALDI.

Disadvantages: limited dynamic range, influenced by space charge effects and ion molecule reactions, artefacts such as harmonics and sidebands are present in the mass spectra

● **Time of flight (TOF)**

It is the simplest mass analyzer, based on the principle that when molecules are given the same amount of energy, they migrate along a drift zone to a detector within different times, according to their mass.

The equation governing TOF separation is:

$$\frac{m}{z} = 2eEs \left(\frac{t}{d} \right)^2$$

Where:

E is the extraction pulse potential

s is the length of flight tube over which E is applied

d is the length of field free drift zone

t is the measured time-of-flight of the ion

At the end of the drift zone a series of electric field (reflectron) are applied to re-pulse the ions back along the flight tube. The aim is to solve the problem of the existence, for each discrete m/z , of a distribution of kinetic energy due to the fact that the pulse is not felt by all ions to the same intensity.

At first TOF analyzers were coupled with a pulsed ion source, as MALDI, because ions have to enter in the analyzer at the same time; nowadays electrospray has also been adapted where the ions from the continuous ESI source can be stored in the hexapole (or octapole) ion guide then pulsed into the TOF analyzer. Thus, the necessary electrostatic pulsing creates a time zero from which the TOF measurements can begin.

Characteristics

Accuracy: 200 ppm, 10 ppm with reflectron

Resolution: 8 000, 15 000 with reflectron

m/z range: 4 000

Scan speed: milliseconds

Tandem mass: only with reflectron, by taking advantage of MALDI fragmentation.

The reflectron takes advantage of the fact that the fragment ions have different

kinetic energies and separates them based on how deeply the ions penetrate the reflectron field, thus producing a fragment ion spectrum

It can be coupled it with MALDI, ESI and Gas Chromatography and it present an extreme sensitivity.

Disadvantages: fast digitizers used in TOF can have limited dynamic range, limited precursor-ion selectivity for most MS/MS experiments.

➤ **Quad-TOF mass analyzer**

It combines the stability of a quadrupole analyzer with the high efficiency, sensitivity, and accuracy of a time-of-flight reflectron mass analyzer. The quadrupole can act as a simple analyzer that scan a specific mass range or can select a particular ion that will be fragmented in the collision cell; the TOF analyzer has the great capability to achieve simultaneous and accurate measurements of ions across the full mass range.

Characteristics

Accuracy: 10 ppm

Resolution: 10 000

m/z range: 10 000

Scan speed: ~a second

Tandem mass: MSMS, excellent accuracy, good resolution. Low energy collision and high sensitivity

Ion detector

○ **Faraday cup or cylinder**

It uses a dynode electrode made of a secondary emitting material like CsSb, GaP or BeO.

The ions strike the dynode surface which emits electrons, creating a positive charge on the detector and so induced a current which is amplified and recorded.

It is not so sensitive but it is very robust, recommended for isotope analysis and IRMS.

○ **The electron multiplier**

It is an extending of the Faraday Cup: the first type of multiplier is made up of a series (12 to 24) of Aluminium Oxide dynodes maintained at increasing potential and resulting in a series of amplifications.

The second type, called "Channel Multiplier" has a curved continuous dynode where ion collision with the dynode surface generate amplifications. The secondary ions so generated, are attracted either to the second dynode held at higher potential, or into the continuous dynode generating a repeated process that ultimates in a cascade of electrons.

Advantages: It is very useful especially when positive and negative ions need to be detected in the same instrument, amplification of the order of 1 million to 1.

○ **The photomultiplier or scintillation counter**

In this way the secondary electrons, emitted after the ion impact with a dynode, strike a phosphorous screen which in turn release a burst of photons. Then these photons are amplified through a photomultiplier in a cascade fashion.

Advantages: the multiplier can be kept sealed in a vacuum preventing contaminations and extending the lifetime of the detector.

- **Array detector**

Typically used with magnetic sectors, it is made by a group of individual detectors, aligned in an array format, that spatially detect ions according to their different m/z .

Advantages: scanning is not necessary and therefore sensitivity is improved (over a small mass range).

- **Charge (or inductive) detector**

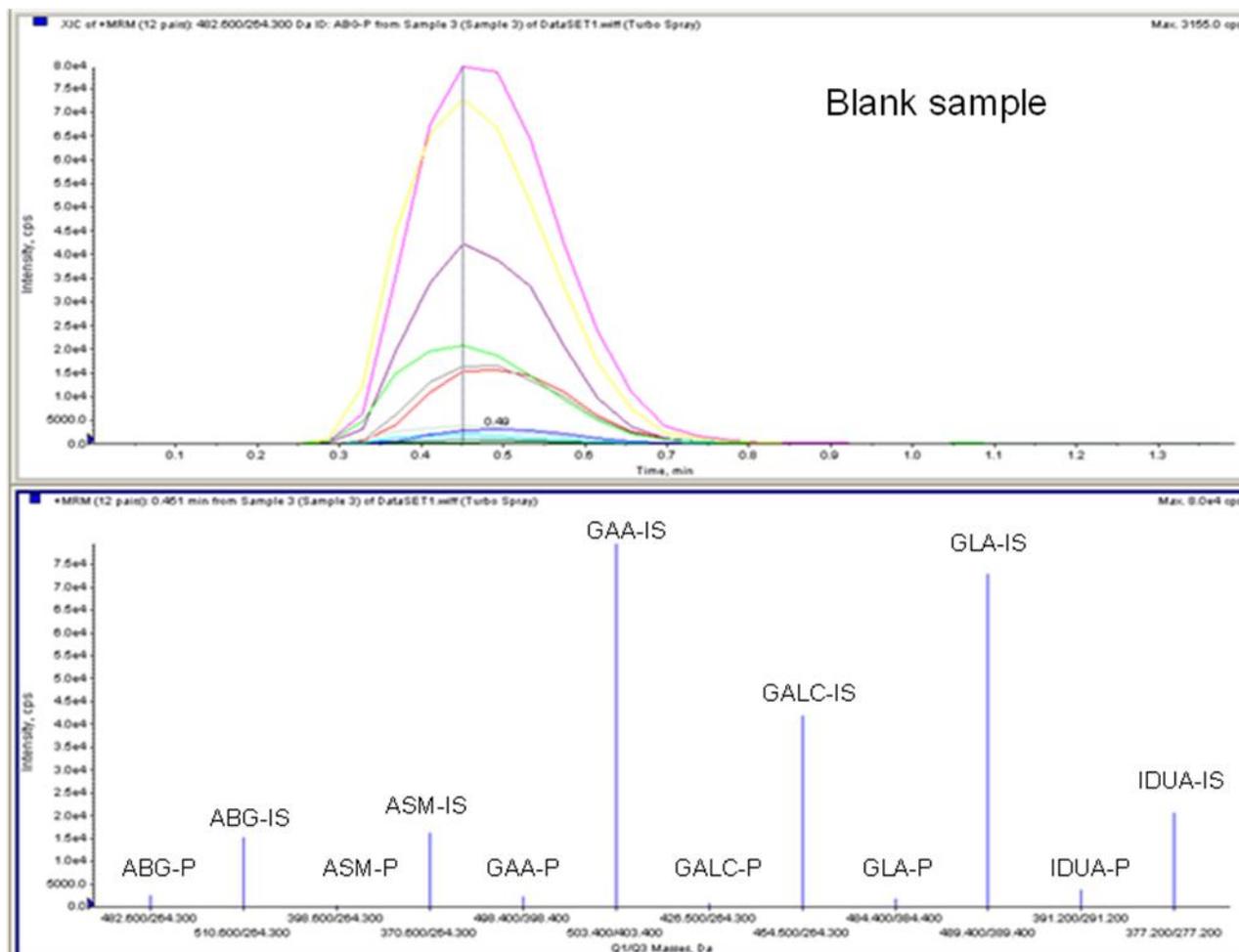
It is widely used in FTMS to generate an image current of an ion.

It simply recognizes a moving charge particle that passes through a plate and induces a current on the plate

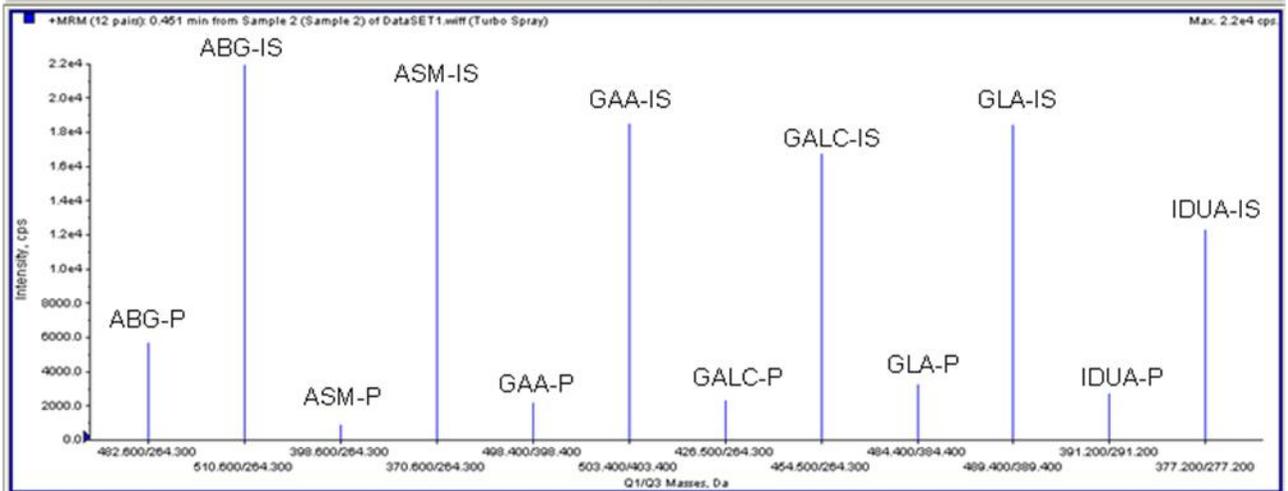
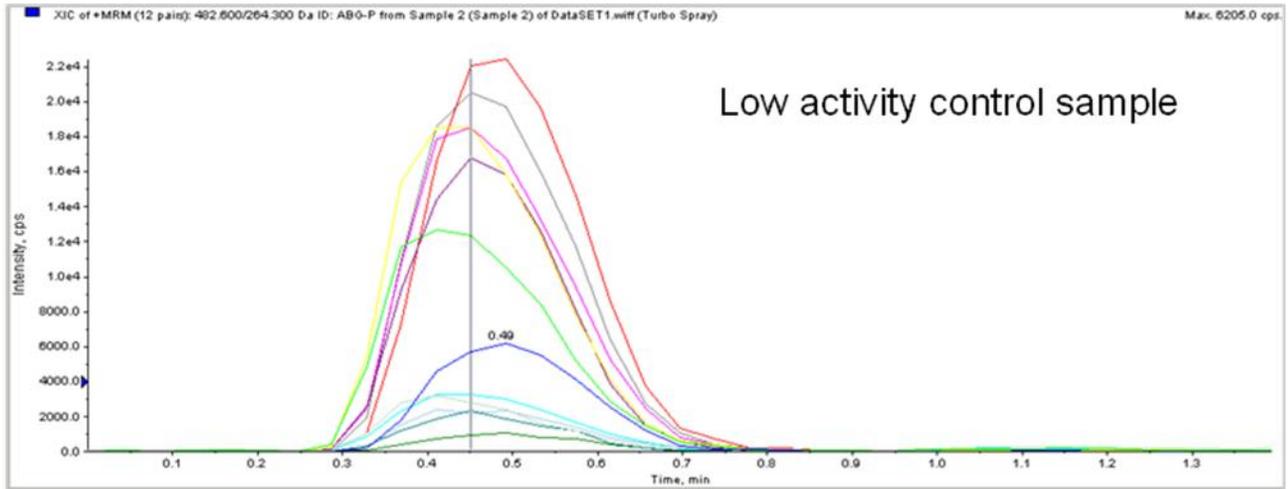
Advantages: independent of ion size

Appendix B

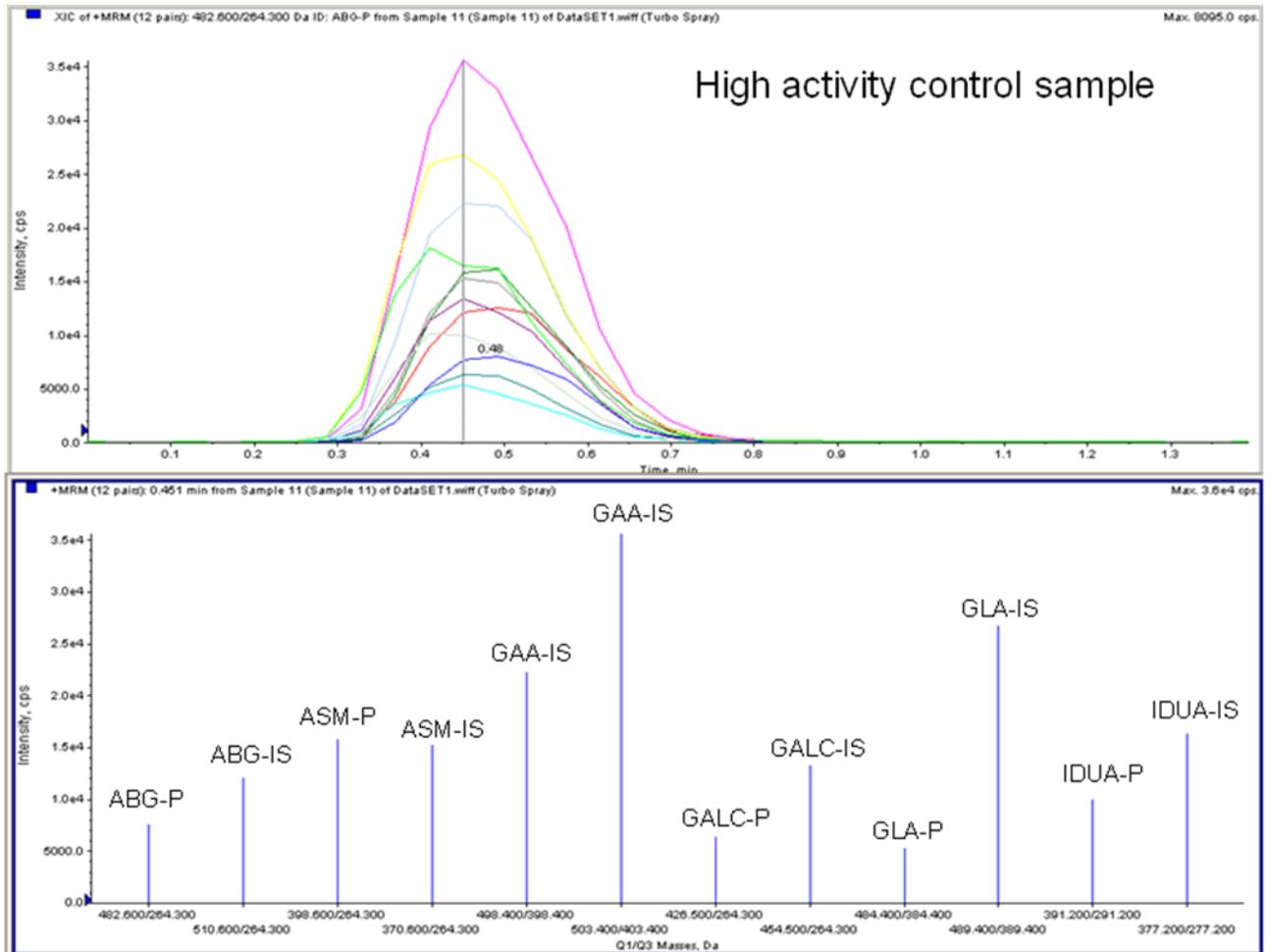
Blank sample. Products and Internal Standards signals.



Low activity control sample. Products and Internal Standards signals



High activity control sample. Products and Internal Standards signals



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