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Short communication

The inclusion of ADA-SCID in expanded newborn screening by tandem mass spectrometry

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ABSTRACT

Severe combined immunodeficiency due to adenosine-deaminase defect (ADA-SCID) is usually deadly in childhood because of severe recurrent infections. When clinical diagnosis is done, permanent damages due to infections or metabolite accumulation are often present. Gene therapy, bone marrow transplantation or enzyme replacement therapy may be effective if started early. The aim of this study was to set-up a robust method suitable for screening with a minimized preparation process and with inexpensive running costs, for diagnosing ADA-SCID by tandem mass spectrometry. ADA-SCID satisfies all the criteria for inclusion in a newborn screening program.

We describe a protocol revised to incorporate adenosine and 2-deoxyadenosine testing into an expanded newborn screening program. We assessed the effectiveness of this approach testing dried blood spots from 4 genetically confirmed early-onset and 5 delayed-onset ADA-SCID patients. Reference values were established on 50,000 healthy newborns (deoxyadenosine <0.09 μmol/L, adenosine <1.61 μmol/L). We also developed a second tier test to distinguish true positives from false positives and improve the positive predictive value of an initial abnormal result.

In the first 18 months, the pilot project has identified a newborn with a genetically confirmed defect in adenosine deaminase (ADA) gene.

The results show that the method having great simplicity, low cost and low process preparations can be fully applicable to a mass screening program.

1. Introduction

Severe combined immunodeficiency due to a defect of adenosine deaminase (ADA-SCID, OMIM # 102,700) is an inherited disorder of purine metabolism [1] representing for approximately 15% of all SCID cases and one-third of cases of autosomal recessive SCID [2]. It is caused by mutation in the gene that encodes a protein called adenosine deaminase (ADA, EC 3.5.4.4) [3]. Deficiency of the purine salvage enzyme ADA results in varying degrees of immunodeficiency, ranging from neonatal onset to late onset immunodeficiency which can determine severe impairment of lung function in adolescents or adults [4]. About 85–90% of patients show an early severe SCID, that in the absence of treatment, leads to death within the first year of life. Onset occurs between 6 months and the first few years in 10–15% of patients with a 'delayed' clinical manifestation and from second to fourth decades in a smaller percentage of patients with 'late' onset, showing less severe infections and a slowly deteriorating immune function [5]. Finally, the benign condition called 'partial' ADA deficiency is characterized by a decreased enzyme activity in erythrocytes, but a residual enzyme activity in leukocytes and other nucleated cells [6].

In the early onset form, the severe functional defect of the enzyme ADA leads to the accumulation of toxic metabolites,

Abbreviations: ADA-SCID, severe combined immunodeficiency due to adenosine-deaminase defect; SCID, severe combined immunodeficiency; ADA, adenosine deaminase; dAdo, 2-deoxyadenosine; ERT, enzyme replacement therapy; Ado, adenosine; TRECs, T-cell receptor excision circles; DBS, dried blood spot; HPLC, high-performance liquid chromatography; RT, room temperature; LOD, limit of detection; LLOQ, lower limit of quantitation; SUAC, succinylacetone; MRM, multiple reaction monitoring; LC–MS/MS, liquid chromatography/tandem mass spectrometry; MS, mass spectrometry.

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represented primarily by 2-deoxyadenosine (dAdo) and dATP, the phosphorylated end product of the ADA substrate dAdo [7]; on turn increased dAdo levels inactivate the enzyme S-adenosylhomocysteine hydrolase and result in inhibition of transmethylation of nucleic acids, proteins, and lipids [8].

Treatment options for ADA SCID are represented by stem-cell transplantation, enzyme replacement therapy (ERT) and gene therapy. Haematopoietic stem cell transplantation is today the gold standard treatment for ADA-SCID, although it is dependent on a good donor match [9]. ERT with polyethylene glycol-modified bovine adenosine deaminase (PEG-ADA, Adagen®) is available and determines the elimination of toxic metabolites and a good reconstitution of the immune system [10]. Gene therapy has also shown promising results for some patients with ADA deficiency SCID [11].

In any case, an early treatment is mandatory to prevent ADA SCID symptoms and also to get the best therapeutic effect. For this reason, diagnostic methods to make early diagnosis are important to significantly improve patient health outcomes, while at the same time reducing the cost of medical care.

Diagnosis can be made detecting either ADA enzyme activity or accumulation of metabolites due to ADA deficiency.

Evaluating ADA activity is complex and sometimes can give misleading results: actually a severe defect in ADA activity can be found in clinically unaffected subjects with a 'partial' ADA deficiency, because variable residual ADA activity expressed in cells different from immune cells can be sufficient to maintain good immune function [12,13]. Therefore measurement of metabolites is absolutely mandatory to confirm the diagnosis of immunodeficiency due to ADA deficiency. Moreover dosage of metabolites allows monitoring the reduction of their levels after therapy. dAdo and adenosine (Ado) quantification are important in the diagnosis of ADA SCID and their measurement in urine samples is performed in clinical practice by different methods, ranging from HPLC to thin-layer chromatography, capillary electrophoresis and even reverse-phase HPLC with electrospray ionization tandem mass spectrometry [14–17]. Anyway these methods are used when an immunodeficiency is suspected, generally when it's too late to prevent the damage and there is the risk of permanent disability.

Newborn screening for SCID based on measurement of T-cell receptor excision circles (TRECs), is able to identify all T lymphocytes defects, and was started in Wisconsin in 2008. It has been adopted in part of the United States and in many countries around the world since January 2010, when Secretary Advisory Committee for Heritable Disorders in Newborns and Children recommended the inclusion of SCID in the newborn screening panels [18].

Mass spectrometry is extensively used in newborn screening programs for analysis of metabolites from dried blood spots (DBS) collected a few days after birth, but among the detected metabolites those due to ADA deficiency have not been included so far [19,20].

Aim of the present work was to provide an analytical method that could allow the quantitation of ADA-SCID metabolites at the same time with the other metabolites that are commonly determined in expanded newborn screening programs.

2. Materials and methods

2.1. Standards and chemicals

Ado and dAdo were purchased from Sigma Aldrich (St. Louis, MO, USA); Adenosine-ribose-1-^{13}C (^{13}C, 99%) and deoxyadenosine-ribose-5-^{13}C (^{13}C, 98%) used as internal standards (IS) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Stock solutions of Ado and dAdo chemical and labeled standard at 100 μmol/L were prepared in HPLC grade water and stored at −20 °C. Working solutions were daily prepared from stock solutions.

2.2. Newborn screening pilot project for ADA-SCID

Blood collection for expanded newborn screening by tandem mass spectrometry (LC–MS/MS) in Tuscany is recommended between 48 and 72 h of life. Blood samples are routinely obtained by heel stick, spotted on filter paper (903®, Whatman GmbH, Dasel, Germany), dried and sent by courier to the screening center.

The method was applied to 50,000 DBS from neonates born in the period between January 2011 and June 2012 and data were used to set up cut-off values. In the first 6 months of the pilot project, DBS were processed in parallel by both the methods reported by la Marca et al. [20] and the new screening method reported by Azzari et al. [21] which is performed without butylation procedure and with the addiction of Ado and dAdo IS, both at 1 μmol/L for each well.

Briefly, 200 μL of methanolic solution of labeled acylcarnitines, aminoacids, SUAC, Ado and dAdo were added to a 3.2 mm-paper circle from the DBS (containing about 3.4 μL of blood). 100 μL of a 3 mmol/L solution of hydrazine in water was added to the mixture. Mixture was put on an orbital shaker and kept at 37 °C. After 25 min, the mixture was dried under N_2 and reconstituted with 300 μL of a mixture of CH_3CN 70% containing 0.1% of formic acid.

2.3. Second tier test

Calibrators were prepared by spotting 25 μL of whole blood from healthy adult blood donor on filter paper for the study of the metabolite stability and for the repeatability tests.

DBSs were dried for a period of at the least 2 h at room temperature (RT) and were stored at 4 °C until analysis in sealed plastic bags containing desiccant and a humidity indicator card. Whole blood from healthy adult donors was collected after they gave informed consent. All experiments were conducted in compliance with institutional review board guidelines (regional pilot project protocol n° 7949/2011).

Calibrators were prepared at 0.1, 1, 10 and 100 μmol/L of dAdo and 0.2, 1, 10 and 100 μmol/L of Ado as follows: a 3.2 mm disk from a DBS was punched and extracted by dispensing 300 μL of a mixture of methanol and water (2:1, v/v) containing ribose-1-^{13}C-adenosine and $^{13}C_5$ deoxyadenosine (10 μmol/L) as internal standard and different concentrations of Ado and dAdo. Calibrators were shaken on a vortex system for 25 min at 37 °C and then transferred to a 96-well plate.

3 μL of each calibrator was injected into the mass spectrometer for the MS/MS experiments. An Applied Biosystems/MDS Sciex API 4000™ triple-quadrupole mass spectrometer equipped with a TurboV-Spray® source with the turbo gas temperature set at 425 °C was used to perform experiments. The source operates in positive ionization polarity at a potential of +5500 V.

A Synergi® fusion RP column (150 mm × 2 mm i.d.; 4 μm particle size) was used for the analysis. The mobile phase composed of a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile was applied in a constant ratio of 20:80 (v/v) at a flow rate of 0.2 ml/min. Retention time of dAdo, Ado and their labeled standards was 1.8 min. The analyte concentrations are calculated automatically using the software Analyst Quantitate (ABI SCIEX, Foster City, CA, USA).

The method was validated in terms of linearity, intra-day and inter-day precision, accuracy by analyzing calibrators prepared as above described. Intra-day precision data were evaluated by ten replicate analysis of four different dAdo and Ado concentrations on the same day. Inter-day precision data were determined by the analysis of four different dAdo and Ado concentrations on 6

different days. Sensitivity was assessed by calculating the limit of detection (LOD) and the lower limit of quantification (LLOQ). They were set by analyzing calibrators at 0, 0.05, 0.1, 0.2, 0.3, 0.5, 0.75, 1 and 1.5 μmol/L both for Ado and dAdo.

Isotope dilution-based quantization was performed by comparing the extracted ion chromatogram (peak area) of the chemical standards and the one from the labeled standards.

2.4. Patients

For the validation of this method, we retrospectively analyzed newborn screening cards from 4 genetically confirmed early onset and 5 delayed-onset ADA-SCID patients. Three were born in Tuscany before ADA-SCID was included in the newborn screening panel and six were from other screening laboratories. Clinical and genetics details on ADA-SCID patients and the correlation of enzyme residual activity and metabolite' levels have been described by la Marca et al. [23].

3. Results

3.1. Newborn screening pilot project for ADA-SCID

The method described herein can be used to extract Ado and dAdo from a DBS sample under conditions that permit concurrently extracting other metabolites, such as succinylacetone (SUAC), amino acids, free carnitine, or acylcarnitines by the use of a mixture of water and methanol. Therefore the test can be used in an expanded newborn screening program or during enzyme replacement therapy to monitor the efficacy of therapy. The traditional screening method was modified by adding labeled Ado and dAdo as internal standards to the methanolic solution of labeled acylcarnitines, amino acids and SUAC.

Mass spectral data for Ado and dAdo were obtained by a Multiple Reaction Monitoring (MRM). Specific MRM transitions for Ado and ribose-1-^{13}C-adenosine (268.2 > 136.1 and 269.2 > 136.1, respectively), and for dAdo, ribose-5-^{13}C deoxyadenosine, (respectively 252.2 > 136.1 and 257.2 > 136.1) were recorded.

Purines are not soluble in pure methanol; the experimental minimum percentage (v/v %) of water in the extracting mixture must be 20%; 35–40% would gives the best results for purines extraction. The presence of hydrazine water solution in the reported extraction procedure (see Section 2) at 33% (v/v) is the best compromise to obtain an optimal extraction yield of both purines and all the other metabolites routinely tested.

The well plate routinely prepared contained a blank filter paper punch and a positive DBS control containing Ado and dAdo at known concentration.

During the screening analysis, the concentration of each analyte was determined through comparison with the internal standard followed by subtraction of the blank value. Validation parameters are reported in literature [21,22].

The new test was applied to more than 50,000 DBS samples from newborns. Table 1 shows cut-off values for Ado and dAdo used for the expanded newborn screening program. No differences between full-term or premature infants were detected. Positive rate at the first screening test was 0.02% (10:50,000 live births); 90% of them (9/10) normalized at the 2nd tier test.

Table 1
Cut-offs of mean ± 4 SD for dAdo and Ado calculated on DBS from 50,000 newborns.

	dAdo	Ado
Mean	0.05	0.56
Median	0.05	0.52
SD	0.01	0.26
Lower cut off (−4SD)	0.00	0.00
Upper cut off (+4SD)	0.09	1.61

Table 2
Intra- and inter-day dAdo reproducibility on DBS assessed by %CV calculations.

Expected concentration (μmol/L)	Mean (n = 10)	SD	%CV	Accuracy (%)
Intraday				
0.10	0.09	0.01	8.10	90
1	0.90	0.05	5.53	90
10	10.21	0.39	3.85	102
100	99.95	5.96	5.96	99
Interday				
0.10	0.09	0.01	13.97	90
1	1.18	0.05	4.36	115
10	10.99	0.32	2.91	109
100	106.41	0.60	0.57	106

We performed retrospective analysis of newborn screening cards from 9 truly positive ADA-SCID patients. All the tested patients were confirmed positive by the test.

An ADA deficient patient, born in Tuscany in 2011, was identified throughout newborn screening pilot project.

Fig. 1 shows the comparison between spectra from an ADA patient and a healthy control.

3.2. Second tier test

Since the inclusion of ADA metabolites in the panel of newborn screening showed some false positives, the development of a second tier test has proved essential to cope the possibility of distinguish true positives from false positives and improve the positive predictive value of an initial abnormal result.

The method was validated, by the use of calibrators at known concentration of ADA metabolites chemical standards, in terms of linearity, intra-day and inter-day precision and accuracy. Calibrators, containing internal standard at 10 μmol/L, were at concentrations of 0.1, 1, 10 and 100 μmol/L for dAdo and at 0.2, 1, 10 and 100 μmol/L for Ado.

Adenosine deaminase is present into the cells as well as on membranes and it catalyzes the irreversible deamination of Ado and dAdo to inosine and deoxyinosine. For this reason standards were not added to the whole blood to prevent ADA metabolism. Calibrators, prepared using a pooled whole blood obtained from healthy donors spotted on filter paper, were extracted as above described. The extracts were enriched of different Ado, dAdo and their internal standard concentrations and were analyzed to test validation parameters.

When real samples from patients were analyzed, methanol based solution deposition was first performed on DBS to prevent enzyme reactivation, then water was used to extract purines.

During the sample preparation procedure, the first step consisted of methanol addition to fix proteins on paper followed by water addiction to extract purines.

The assays were linear up to 100 μmol/L for both metabolites. Intra- and interday imprecision data are reported in Tables 2 and 3. LOD was 0.05 μmol/L for dAdo and Ado and LLOQ was 0.1 μmol/L for dAdo and 0.2 μmol/L for Ado.

In order to establish normal ranges for Ado and dAdo, 250 blood spots, reported as normal during newborn screening by LC–MS/MS, were tested. The reference ranges for the 2nd tier test were <0.1 μmol/L for dAdo and 0.1–2 μmol/L for Ado.

We measured ADA metabolites retrospectively in newborn screening samples of 9 truly positive ADA-SCID patients to assess whether 2nd tier testing could be effective. All the truly positive patients showed ADA metabolites values out of ranges.

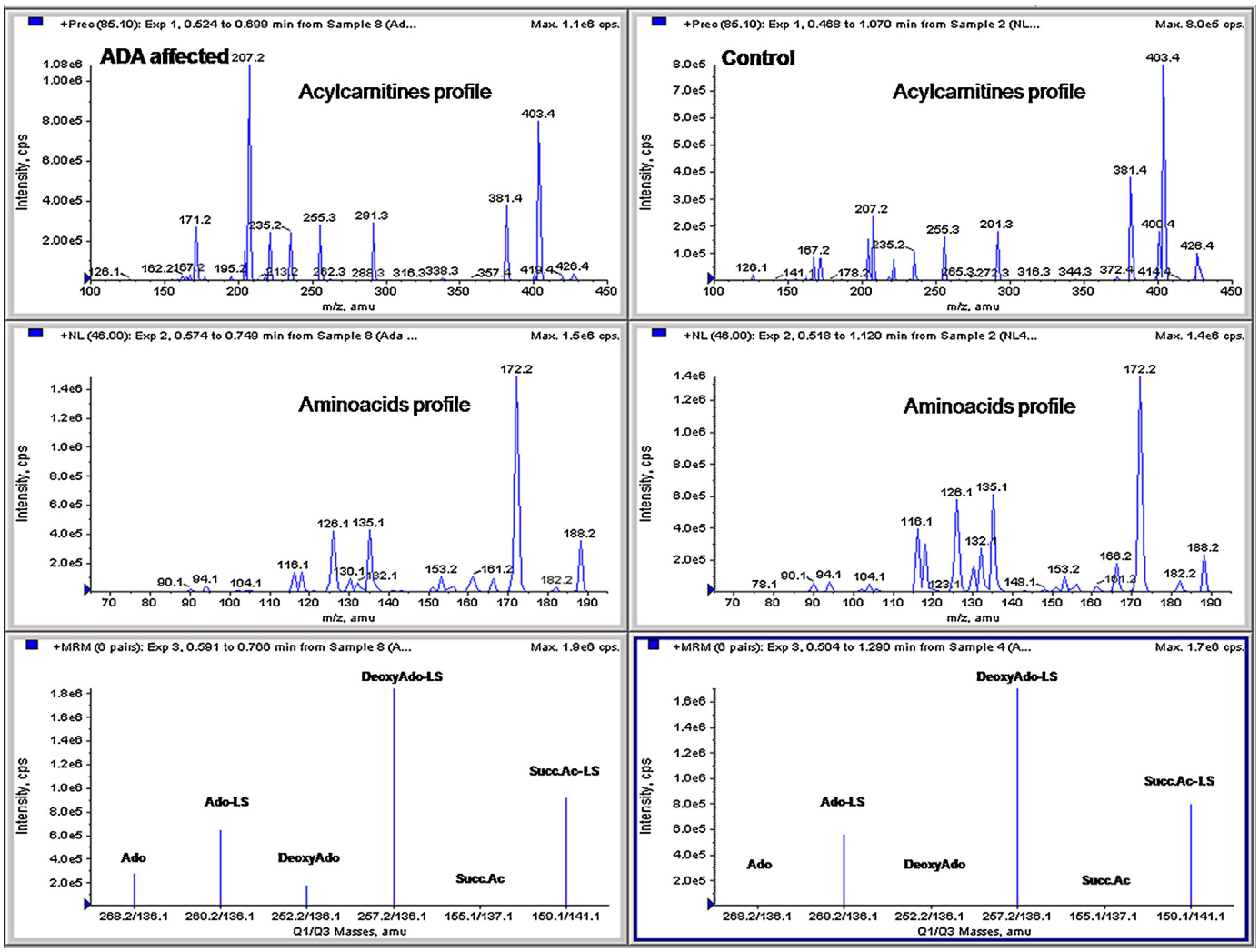

Fig. 1. MRM experiments show dAdo is not present in a control whereas increased levels are observed in a ADA-SCID patient.

Table 3
Intra- and inter-day Ado reproducibility on DBS assessed by %CV calculations.

Expected concentration (μmol/L)	Mean ($n = 10$)	SD	%CV	Accuracy (%)
Intraday				
0.20	0.19	0.03	16.7	95
1	1.15	0.06	4.9	115
10	10.37	0.56	5.4	104
100	99.86	8.07	8.1	100
Interday				
0.20	0.20	0.04	20.62	90
1	1.08	0.02	2.09	108
10	10.46	0.10	0.93	105
100	100.22	0.73	0.73	100

During the newborn screening project 10 spots with abnormal values of dAdo were analyzed by the 2nd tier test. Among them, 9 were found to be false positive and one presented values over normal limits. A second DBS from this newborn was tested and positive results were confirmed. Application of this assay improved the positive predictive value up to 100%. No false negative results have been reported at the moment.

A flow chart used in the pilot screening program for ADA-SCID was developed to apply this method as an efficient and effective second-tier assay on samples with abnormal results by primary screening (Fig. 2).

4. Discussion and conclusions

Up to now, many newborn screening programs worldwide use TRECs from DNA of exhausted DBS as method to evaluate ADA-SCID. Unfortunately, this approach is expensive, it takes a long time and it lacks of sensitivity in diagnosis of late and delayed onset ADA SCID [21,24].

The proposed method can be used to simultaneously extract and quantify Ado and dAdo, amino acids, acylcarnitines and SUAC using LC–MS/MS. Therefore, the inclusion of this test into the expanded newborn screening for inherited metabolic diseases is possible. Considering that no extra operator time was necessary for sample preparation or analysis beside the time necessary for routine neonatal screening, no extra-equipment was necessary and the instruments were not used for longer times, the cost of the test was calculated for reagents only; including unavoidable reagent waste it was valuable in about €0.01 per test.

Some procedures for newborn screening utilize derivatization to form butyl esters and acidified butanol (3 N HCl in *n*-butanol) is added to the extracted DBS in order to improve sensitivity. ADA-SCID inclusion into the newborn screening panel is not compatible with esterification because decreasing the pH leads to degradation of ADA metabolites.

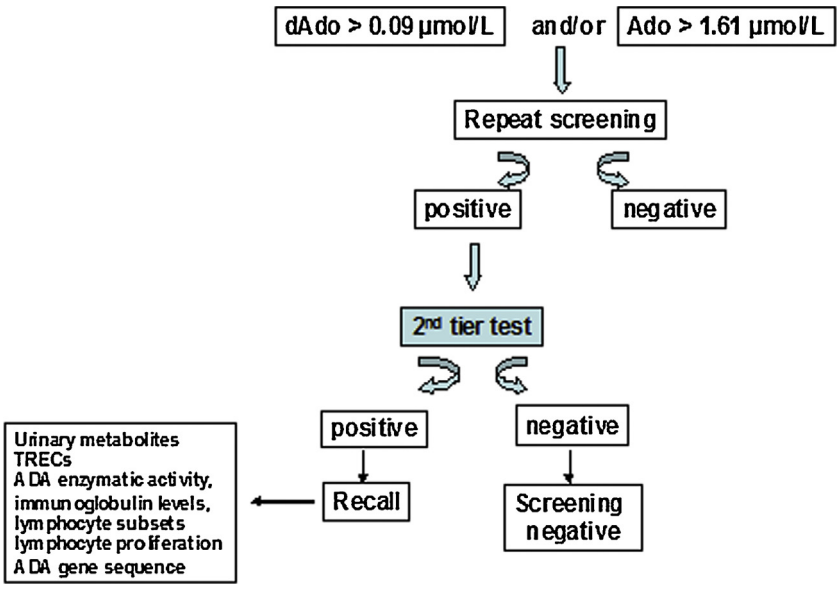

Fig. 2. Flow chart used in the pilot screening program for ADA-SCID.

As an alternative to the proposed underivatized method, a two step procedure could be hypothesized: first step by using methanol as extraction solution for aminoacids and acylcarnitines followed by butilation; second step by using hydrazine solution for SUAC and purines. After evaporation procedure the two reconstituted solutions could be reunified.

Our results show that ADA-SCID can be diagnosed with highest sensitivity and specificity from DBS taken at birth. dAdo is considered the most important marker for ADA-SCID while Ado is the secondary marker.

No extra equipment or time is required beside the time necessary for routine neonatal screening, therefore no additional costs are required for applying such testing. The method is reliable and reproducible, easy to perform and gives a definitive response within a short time.

Moreover an LC–MS/MS based second-tier test was set up in order to reduce false-positive results, which produce unnecessary family anxiety and increase follow-up costs.

Of the 50,000 newborns screened in the first 18 months pilot project, a full-term healthy newborn revealed abnormal levels of both Ado (6.9 μmol/L) and dAdo (1.2 μmol/L).

After positive 2nd tier test result, the newborn was recalled and the confirmatory procedure started.

We were able to confirm that urinary Ado and dAdo levels were increased in the proband using a previously described method [17]. ADA enzymatic activity, immunoglobulin levels, lymphocyte subsets and proliferation as well as ADA gene sequence were performed. Data obtained from all the biochemical and molecular analysis let to include our first patient as possible late onset ADA–SCID [23].

Identification of late onset ADA-SCID disease in newborns may be stressful to the patients and their families; however, the ability to monitor the patients and to perform a correct therapy reduce the risk that irreversible damages occur.

Up to now no later onset ADA-SCID patients have been identified at birth, so no data are present in literature on the natural history of a later phenotype [23]. The present method offers, for the first time, the possibility to get the diagnosis of ADA-SCID at birth, before onset of infectious disease. Therefore, this project also increased our knowledge about the delayed and late manifestation of ADA-SCID disease in young infants.

Early diagnosis of SCID allows treating the affected patients very soon so avoiding severe complications due to infectious disease which are always expected in the follow-up of immunodeficient patients [22].

Considering that at the moment the most part of laboratories performing expanded newborn screening by tandem mass spectrometry are not yet equipped for molecular biology analyses, the present approach has to be considered as a good option to the validated TREC test. Moreover, delayed ADA-SCID false negative cases by TREC test have been recently described [23–25]

Within the last 7 years (2004–2011) approximately 250,000 births occurred in Tuscany, among them four ADA-SCID patients were diagnosed. These preliminary data show the incidence in our catchment area seems to be greater than about 1:500,000 as reported in literature [26].

The proposed method is able to quantify dAdo and Ado during newborn screening; 2nd tier test, as described, reduces recall rate close to zero. In addition, DBS collection has several advantages compared to other frozen specimens including lower cost and easy sample storage and transport. The use of DBS can be a convenient alternative to urines or plasma to make diagnosis of ADA SCID but also to simplify therapy monitoring in patients undergoing bone marrow transplantation, enzyme replacement therapy or gene therapy.

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