Original Article

Detection of acid sphingomyelinase in human saliva and its advantages in the diagnosis of Niemann-Pick disease type B

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Abstract

Background: Niemann-Pick disease type B is an autosomal recessive lysosomal storage disorder caused by a deficiency of acid sphingomyelinase (ASM) coded by SMPD1 gene. Diagnostic assays for this enzyme were developed using fibroblasts, leukocytes, plasma and dry blood spots, however, there are no expression studies in saliva in the literature, so far. Saliva is a biofluid used to analyze the health/disease condition of an individual.

Methods: We standardized a fluorometric method to determine ASM activity in human saliva of control subjects and in one NPD-B patient.

Results: ASM activity was detected in all saliva samples. The range of ASM in saliva of 28 control subjects was 4.5 - 70.4 with an average of 26.93 ± 15.7 nmol/17h/mg of protein. Values in plasma were significantly lower, a 0.056- 3.2 range, with an average of 0.85 ± 0.7 nmol/17h/mg of protein. There was no correlation between saliva and plasma samples (R2= 0.001). ASM was markedly deficient in saliva activity of (0.09 nmol/17h/ mg of protein) as well as in the leukocyte pellet (0.125 nmol/17h/mg protein) and in the plasma (0.68 nmol/17h) of the NPD-B patient.

Conclusion: Our observations indicate that saliva could be an alternative biofluid to plasma and to leucocytes to measure ASM activity, representing a non-invasive, easy-collection diagnostic means, which would allow the identification and characterization of these entities.

Keywords: Acid sphingomyelinase, biomarker, lysosomes, Niemann-Pick disease types A-B

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INTRODUCTION

Acid sphingomyelinase deficiency (ASMD), commonly known as Niemann-Pick disease (NPD), including NPD types A and B, is an autosomal recessive lysosomal storage disorder. The acid sphingomyelinase (ASM) is a glycoprotein that catalyses the breakdown of sphingomyelin into ceramide and phosphocholine mainly

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located in lysosomes. Enzymatic dysfunction of ASM causes NPD type A and B, characterised at the cellular level by the accumulation of sphingomyelin within the endolysosomal compartment. It is coded by the acid sphingomyelin phosphodiesterase 1 (*SMPD1*) gene. The *SMPD1* gene is 5 kb and consists of six exons. It is located on chromosome 11p15.1–11p15.4.[1]

In mammals, two enzymes are formed from a single *SMPD1* gene due to post-translational modifications; the secretory (S-ASM) and lysosomal (L-ASM) forms. Previous studies have shown that L-ASM and S-ASM differ in their molecular weight, their N-terminal amino acid sequence and the presence of complex oligosaccharides. S-ASM is located extracellularly, has a complex N-glycosylation pattern and requires zinc ions for catalytic activity. Conversely, L-ASM is found within the lysosome with high mannose N-glycan oligosaccharides that provide stability and protection within the lysosome and is thus independent of supplemented zinc in biochemical assays.[2,3]

The deficiency of ASM causes accumulation of sphingomyelin in the lysosomes of macrophages and other cell types. ASMD phenotypic variation has been categorised into subtypes based on severity and degree of neurological involvement. The main subtypes are the following: infantile neurovisceral ASMD or NPD type A, which presents the most acute form with early start of central nervous system involvement, marked by growing psychomotor retardation, systemic involvement (hepatosplenomegaly, bleeding and respiratory disease) and early death between 2 and 3 years;[3,4] chronic neurovisceral ASMD (intermediate form; NPD types A/B or NPD type B variant), which is characterised by slower advancement of degenerative neurological conditions and extended survival in comparison to infantile neurovisceral ASMD; chronic visceral ASMD (NPD type B) that presents little or no neurological involvement with progressive hepatosplenomegaly and infiltrative lung disease and is characterised by survival until adulthood.[5]

Although no effective treatment to halt disease progression has been found so far, early diagnosis increases prognosis through opportune control of unfavourable evolution. Enzyme replacement therapy (ERT) with recombinant sphingomyelinase is presently on trial as a prospective treatment for NPD type B patients through infusion of recombinant ASM. The efficacy in the clinical trials with ERT in other lysosomal diseases suggests that this therapeutic approach may be very effective for NPD type B patients; however, ERT with olipudase alfa is in clinical development at present.[6,7]

Among the spectrum of biofluids that help in the diagnoses and prognosis of diseases is human saliva. Saliva is an oral biofluid that has become an appealing option to obtain biological samples from patients suffering from different disorders. Since it contains specific soluble biological markers (biomarkers), it is useful for the analyses of the medical condition of an individual, for laboratory and clinical diagnosis, for planning approaches to prognosis and for patient monitoring and management as well.[8-11] Saliva has been proved to be an excellent diagnostic tool for a broad spectrum of diseases, such as cardiovascular diseases, autoimmune diseases, kidney failure, viral and bacterial infections, oral disorders such as periodontal diseases, oral cancers[12,13] as well as inherited metabolic diseases, among them neuronal ceroid lipofuscinoses[14] and the defect deficiency of creatine.[15]

Our aim was, first, to determine if ASM could be detected in the human saliva of healthy subjects. To achieve this goal, we standardised a fluorometric method to determine ASM activity in the human saliva of control subjects. Second, once the healthy population reference range of this enzyme activity in that biofluid was established, to validate this method in NPD type B patients.

MATERIAL AND METHODS

We studied the ASM activity in the saliva of 28 control subjects (age range 21–57 years), 1 NPD type B patient and her heterozygous mother. The patient was diagnosed at the Children's Hospital of Córdoba, Argentina, according to the diagnostic algorithm (Figure 1). We followed the procedures in the current revision of the Helsinki Declaration, and we obtained informed consents from the institutional review boards of CIEIS of Polo Hospitalario-Ethics Committee, Children's Hospital, Córdoba, Argentina.

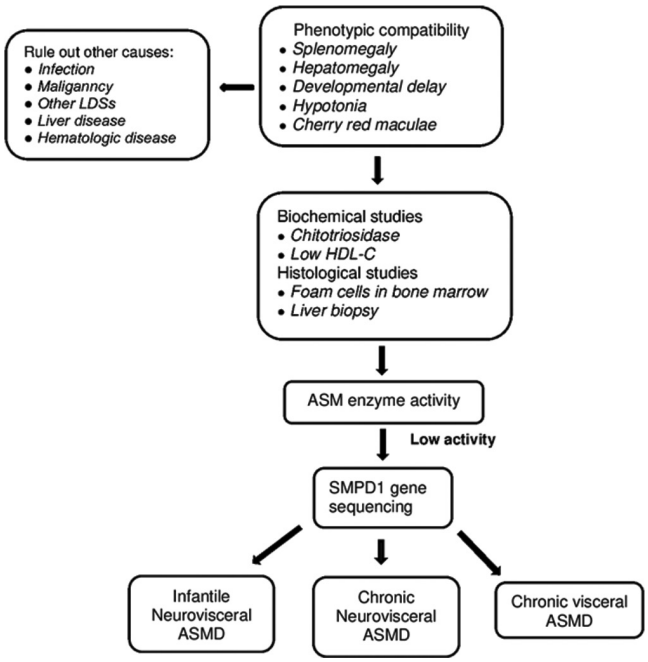

Figure 1: Diagnostic algorithm for acid sphingomyelinase deficiency. LDSs = lysosomal storage diseases; HDL-C = high-density lipoproteins cholesterol; SMPD1 = acid sphingomyelin phosphodiesterase 1 gene; ASMD = Acid sphingomyelinase deficiency

The unstimulated whole saliva was collected by passive spitting into Eppendorf tubes and then placed on crushed ice. To minimise circadian rhythm influences, all salivary samples were collected between 9 a.m. and 11 a.m. The saliva samples were promptly centrifuged at 3000 ×*g* for 30 min at 4°C, to remove cells and debris. The supernatants were transferred to an Eppendorf tube, frozen and kept 40°C, until analysed.[14] The saliva and plasma samples were taken on the same day and time. Whole blood was collected in heparin tubes and centrifuged to separate blood components at room temperature. Plasma supernatants were transferred by cryotubes and stored at −40 °C until further analysis.

We adapted a fluorometric enzyme assay for Niemann-Pick (NP) types A-B diagnosis to test this enzyme in the saliva[16] We measured ASM activity in the saliva and plasma using the fluorogenic substrates 6-hexadecanoylamino-4-methylumbelliferyl-phosphorylc holine (HMU-PC). We obtained HMU-PC from Dr van Diggelen, O.P., Department of Clinical Genetics, EUR, the Netherlands, and he provided us with the standardised enzyme analysis protocol. As a source of ASM, we used saliva and plasma samples from healthy controls, and although two patients were diagnosed with NPD-B at the Hospital de Niños de la Santísima Trinidad,Córdoba, Argentina (Children's Hospital), we got saliva samples from only one patient and plasma samples from both patients. The assay contained saliva and plasma (40 μg protein = 20 μL), 0.66 mM HMU-PC in 0.1 M sodium acetate buffer (0.2% [w/v] synthetic sodium taurocholate and 0.02% [w/v] sodium azide, pH 5.2), in a final volume of 60 μL. Reactions were incubated for 17 h at 37 °C , and the reaction was stopped by the addition of 0.5 mL of 0.2 M glycine/NaOH buffer, pH 10.7 + 0.2% SDS + 0.2% Triton x-100. The samples were centrifuged at 2000 ×g at room temperature, and the generation of fluorescent product 4-methylumbelliferone (4-MU) was measured in a LS 50B Perkin Elmer fluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The linearity of the assay in regard to sample volume and incubation time was confirmed using a standard 4-MU calibration curve. ASM activity was expressed as nanomoles of substrates hydrolised per hour per milligram of protein. All determinations were performed in duplicate. Coefficient of variation was 58% and the limited detection was 0.06436, six blanks were used and standard deviation (SD) was multiplied by 3 and divided by the slope of the calibration curve and the limited quantification was 0.21455.

Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS), Version 21 for Microsoft Windows (IBM Corp Somers NY, USA). Results were presented as mean ± SD. For group comparisons, the statistical significance of differences in mean values was determined Student's *t*-test. To perform correlations, we analysed the data using Pearson's correlation. The statistical significance of differences was regarded to be $P < 0.05$.

RESULTS

In this study, 28 control subjects were screened for ASM activity in the saliva which ranged from 4.5 to 70.4 with a mean value of 26.93 ± 15.7 nmol/17h/mg of protein. Values in the plasma were significantly lower, (range 0.056–3.2, with an average of 0.85 ± 0.7 nmol/17h/mg of protein). ASM activity was detected in all saliva samples of healthy controls. In NPD-B patient 1, ASM activity was markedly deficient in the saliva (0.09 nmol/17h/mg of protein; normal range [NR]: 4.5–70.4 nmol/17h/mg of protein), in the leucocyte pellet (0.125 nmol/17h/mg of protein; NR=5.3-47 nmol/17h/mg of protein as well as in the plasma (0.68 nmol/17h/mg of protein; NR=0.056–3.2 nmol/17h/mg of protein). In patient 2, leucocyte pellet value was 0.46 nmol/17h/mg of protein and plasma was 0.13 nmol/17h/mg protein. We could not determine this patient's ASM in the saliva due to lack of family collaboration. The heterozygous mother had an ASM activity of 3.53 nmol/17h/mg of protein

in leucocytes pellet. No significant correlation was found between saliva and plasma samples according to Pearson's correlation coefficient = 0.17, $P = 0.39$ (Table 1). Figure 2 shows the distribution of values of ASM in the plasma and saliva of control subjects and those found in the patients and not correlation between them as we show in Figure 3. The male-female ratio in the group was 2.5 with ages ranging between 21 and 57 years.

Table 1: Acid sphingomyelinase activities in normal control and patient P1/P2 in leucocyte pellet, plasma and saliva

Variable	Age (years)	Saliva* (nmol/17h/mg of protein) (n=28)	Plasma* nmol/17h/mg of protein) (n=28)	Leucocytes* (nmol/17h/mg of protein) (n=3)
Normal control		26.93 ± 15.7	0.85 ± 0.7	6.2 ± 0.8
	21-57	4.5 -70.4	0.056 - 3.2	5.3-6.75
ASM				
P1	56	0.09	0.68	0.125
P2	6	ND	0.13	0.46
Carrier	46	ND	ND	3.53

Pearson's correlation coefficient = 0.17; $P = 0.39$
*Data are presented as mean ± standard deviation
ASM=Acid sphingomyelinase; P=Patient

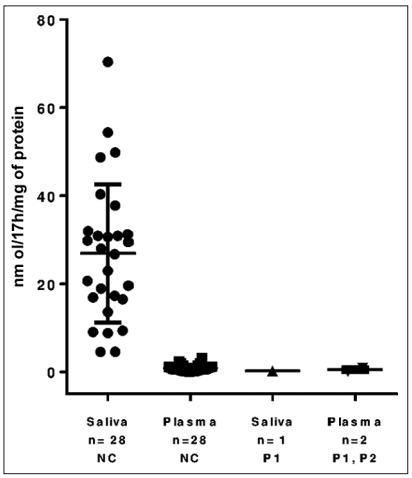

Figure 2: Distribution of enzyme activity in the saliva of healthy controls and deficient subjects. NC = Normal control, P = Patient

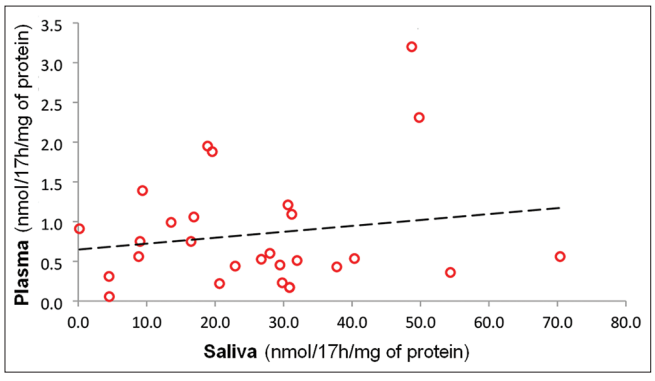

Figure 3: No correlation between acid sphingomyelinase activities in plasma and saliva

DISCUSSION

Nowadays, diverse methods, standards and procedures are employed to diagnose varied diseases. Among them, the utilisation of a spectrum of biofluids including plasma, serum, urine, synovial fluid, tissue homogenates, cerebrospinal fluid (CSF), tear and recently saliva. Saliva provides a number of favourable aspects that have assured the extensive use of metabolites as a tool for diagnosis in clinical practice.[11,17,18]

In this study, saliva and plasma ASM activity has been assayed using the fluorogenic substrate HMU-PC by duplication, the coefficient of variation was 58% and this indicates that the method has good reproducibility. This technique is specific to detect ASM.[16] This is a new fluorogenic enzyme assay for ASM in fibroblasts and leucocytes which detects NP A-B patients, including patients with pseudonormal activity.[16] Previously the distribution of ASM in various human body fluids was studied, observing increased ASM activity in salivary and tear fluids.[19] Little is known about the biological activity and function of S-ASM in the saliva. Very high Zn^{2+}-dependent S-ASM activity has been observed in the saliva, and others[20] suggest that S-ASM may participate in the digestion of the abundant sphingomyelin in the normal diet. They modified a previously described method that differentiates L-ASM from S-ASM forms, but they were unable to observe any ASM activity in CSF. Subsequently, S-ASM activity in human and mouse CSF was detected, quantified and characterise biochemically in human control CSF samples by a sensitive enzyme assay using on a fluorescent substrate.[21] We used similar assay conditions, and the ASM was stable at 37 °C; however, they used a much lower sample volume than ours (sample volume of 1–2 μL) within 24 h incubation, S-ASM activities were between pH = 5.0 and 5.6 and the S-ASM activity was related by type and concentration of Nonidet and triton X-100 detergent with addition Zn^{2+}. A more sensitive method was published.[22] using tandem mass spectrometry and an artificial substrate called short-chain C6-sphingomyelin for pilot screening study of patients with NPD types A and B. Besides, they increased the sodium taurocholate detergent concentration assay buffer and observed a better separation in ASM activity between normal subjects and NPD patients.

In our study, we used van Diggelen's method,[16] which he describes as 'simple, rapid and robust', for the determination of ASM activity in the saliva and plasma. In this method, no Zn^{2+} is added in the fraction of assay; therefore, it does not discriminate the L-ASM and S-ASM; however, being

ASM present in this extracellular fluid, it is inferred that it is the ASM form. We observed that the ASM activity in saliva was higher than in the plasma; a similar result was observed in plasma with low control activity (1.8–13 nmol/17 h per mL; $n = 3$) and poor discrimination of NP types A-B patients (0.7–1.5; $n = 3$).[16]

Lately, saliva has emerged as a valuable alternative to obtain biological samples from patients since it provides several significant advantages. In relation to the patient, particularly children or psychiatric patients, the collection of this biofluid is non-invasive and painless; therefore, it can be collected with little discomfort and lower anxiety than blood draw or less difficulty and distress than generating urine samples. On the other hand, it is a low-cost alternative of easy collection, handling and storage.[23] In addition, saliva sample contains an important source of cells and DNA, protein and circulating microRNA.[11,13,18,24]

The aim of this study was to evaluate the level of ASM in saliva, which may be considered a biomarker in patients with clinical manifestations of NPD types A and B. The result obtained indicated that salivary ASM can be used as a biomarker for detection, monitoring and treatment of ASMD (NPD type A-B).

The adapted method for the determination of activity of ASM in the human saliva is sensitive and precise. This finding indicates that saliva, characterised by being a non-invasive, easy collection, handling and storage and inexpensive means, is a beneficial alternative biofluid to plasma and to leucocytes to measure ASM activity in NPD.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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