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Association of gingival crevicular fluid and serum intracytoplasmic enzyme levels in periodontally healthy homozygous (major) β-thalassemia patients

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Abstract

Objective: To assess tissue necrosis in β-thalassemia major patients, as in other areas of medicine, lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) have been widely used. These markers of tissue degradation have also been studied in the gingival crevicular fluid (GCF) in relation to periodontal disease status. The purpose of this study was to investigate whether periodontal diagnostic tests based on these markers of tissue degradation are influenced from the enzymes' levels in serum and, therefore, could be used in the assessment of the patient's periodontal status.

Material and Methods: Forty-four periodontally healthy, homozygous β-thalassemia patients were enrolled in the study. GCF and serum samples were obtained and the levels of AST, ALT and LDH were determined; the measurements took place in an automated analyzer (Hitachi 777) using the kits of Roche Company.

Results: Lack of correlations between serum and GCF enzyme levels was demonstrated. Serum LDH and serum AST, serum AST and serum ALT are significantly positively correlated. Concerning the GCF, AST and ALT were proved to be significantly positively correlated.

Conclusions: Elevated values of LDH, ALT and AST in serum do not constitute a confounding factor in GCF measurements of the respective enzymes.

Key words: alanine aminotransferase; aspartate aminotransferase; β-thalassemia; gingival crevicular fluid; lactate dehydrogenase; periodontal health

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β-thalassemia is a chronic, familial, hemolytic anemia occurring in populations from countries bordering the Mediterranean and from Southeast Asia (Caffey 1957, Poyton & Davey 1968). Three phenotypic classes of β-thalassemia have been defined: major, intermedia and minor. And they are associated to multiple complex genetic mutations (Schwartz et al. 1995).

Homozygous (major) β-thalassemia patients exhibit accumulation of unpaired α-chains that precipitate within red blood cell precursors as inclusion bodies, leading to oxidative membrane changes and death of the precursor cells in the bone marrow or rapid sequestration within the spleen. Thus, erythropoiesis is rendered ineffective, resulting in severe chronic hemolytic anemia and hypoxia (Piomelli & Loew 1991). Because hemoglobin level (< 7 g/dl) is too low to be compatible with normal development, standard therapy comprises of hypertransfusion of blood to maintain the level of hemoglobin between 10 and 14 g/dl. Usual complications involve iron accumulation, primarily within the hepatic and cardiac parenchyma, leading to hemochromatosis, and subsequently liver and myocardial dysfunction (Schwartz et al. 1995).

To assess tissue necrosis in β-thalassemia major patients, as in other areas of medicine (La Due et al. 1954, DeRitis et al. 1957, Wroblewski & Gregory 1961, Nakamura et al. 1967, Bauer et al. 1974, Wallach 1978, Adolph & Lorenz 1982, Blatt et al.

1982), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) have been widely used, because they comprise soluble intracytoplasmic enzymes, confined to the cytoplasm but released on cell death. Serum LDH is mildly elevated because of ineffective erythropoiesis and has been used to monitor intramedullary hemolysis (Toren et al. 1996). Serum AST and ALT have been employed to evaluate the level of hepatic and cardiac functions (Hoffbrand et al. 1979, Wolfe et al. 1985, Triadou et al. 1989, Di Marco et al. 1993, De Simone et al. 1997).

These markers of tissue degradation have also been studied in the gingival crevicular fluid (GCF) in relation to periodontal disease status, because cell death is an integral and essential component of periodontal tissue destruction (Snyder & Wolff, 1983, Chambers et al. 1984, Lamster et al. 1990, Persson et al. 1990a, b, Smith & Geegan 1991, Persson & Page 1992, 1990, Nakamura et al. 2000, Shimada et al. 2000, 1999, Tsalikis et al. 2001). As the GCF is an osmotically mediated inflammatory exudate increasing with inflammation and capillary permeability, serum is the primary source of its aqueous and proteinaceous component (Tollefsen & Saltvedt 1980, Cimasoni 1983), but contains also non-serum proteins collected en route from the local inflammatory reaction (Johnson 1991, Page 1992, Lamster et al. 1994, McCulloch 1994). GCF levels of these enzymes should provide evidence of cell death within the periodontal tissues and possibly disease activity (Eley & Cox 1998). However, in patients with major β-thalassemia, serum LDH, AST and ALT levels may confound respective GCF enzyme activity.

The purpose of this study was to examine any possible correlations between LDH, AST and ALT levels in serum and GCF in periodontally healthy β-thalassemia major patients and, consequently, investigate whether periodontal diagnostic tests based on such markers of tissue degradation are influenced from the enzymes' levels in serum and if therefore could be used in the assessment of the patient's periodontal status.

Materials and Methods

Forty-four non-smoking Greek patients aged 21–35 years, 20 males and 24 females, suffering from homozygous (major) β-thalassemia and seeking treatment at the AHEPA General Hospital at the Aristotle University of Thessaloniki were enrolled in the study. All subjects were either periodontally healthy, or suffering from moderate gingivitis. Patients in pregnancy, or suffering from other systematic diseases and those who had used antibiotics during the previous 6 months were excluded from the study.

At the first visit a periodontal examination, which included measurements of pocket depth (PD), attachment level (AL) and bleeding upon probing (BOP) was undertaken. A full-mouth X-ray status was obtained to confirm the initial diagnosis. The patients then received oral hygiene instructions, all overstanding fillings and crown margins were polished, supra- and subgingival calculus were removed. The patients' motivation and instruction was continued till the oral hygiene was satisfactory (O'Leary Dental Plaque Index $< 10\%$) (O'Leary et al. 1972) and clinical signs of gingivitis disappeared. No rinsing with antimicrobial agents was administered.

After establishment of periodontal health, the GCF samples were obtained from the mesio-buccal sites of the Ramfjord teeth of each patient. The GCF collection sites were isolated with cotton rolls, air-dried and any supragingival deposits present were carefully removed in order to avoid any contamination with supragingival plaque and saliva. Sterile paper strips (Periopaper®, Pro Flow™ Inc., Amitiville, NY, USA) were gently placed at the gingival crevice for 30 s and afterwards were placed in elastic tubes containing 300 μl Tris-HCl, and after 24 h agitation at 4°C they were removed, the samples were centrifuged and the eluates were frozen at -70°C till they were analyzed. It has been shown that no protein composition alterations occur at this temperature within 6 months (Curtis et al. 1988). The strips were then placed in one tube per patient and therefore the samples are described as pooled. The serum samples were obtained immediately after the GCF collection.

The levels of AST, ALT and LDH in serum and GCF were determined; the measurements took place in an automated analyzer (Hitachi 717, Hitachi, Tokyo, Japan) using the kits of Roche Company (Basel, Switzerland).

The AST measurements were based on the establishment absorbency coefficient of NADH. The reaction was initiated by the addition of patient sample to reagent. The enzyme AST catalyzes this equilibrium reaction. The increase in oxaloacetate is determined in an indicator reaction catalyzed by malate dehydrogenase (MDH).

The rate of decrease in the concentration of NADH is directly proportional to the AST activity in the sample. Absorbance data are converted into reportable activity values based on calculations for zero-order kinetics. Pyroxidal 5-phosphate is the coenzyme in the AST reaction (International Federation of Clinical Chemistry 1978). Its presence in the reagent activates any available unsaturated apoenzyme (enzyme without coenzyme) and increases the measurement AST activity.

ALT is the enzyme which catalyzes a known equilibrium reaction. The pyruvate increase is measured in a subsequent indicator reaction which is catalyzed by LDH. In a second reaction, NADH is oxidized to NAD. The rate of decrease in NADH (measured photometrically) is directly proportional to the rate of formation of pyruvate, and thus the ALT activity.

Additionally, the previous test principle, based in the latter reaction, was used for the LDH measurement, because the rate of decrease in NADH is directly proportional to the LDH activity and it is determined photometrically.

According to the manufacturer's specifications, the sensitivity limit of the above assays is 0.

Statistical analysis

The parameters obtained were statistically analyzed by means of Kolmogorov–Smirnov tests of normality and Kendall's τ–b correlations. The statistical package SPSS-10 was used.

Results

All subjects completed the 4-week trial. The motivation and oral hygiene instructions of the participants, which were undertaken during the pre-study period, ensured that absence of microbial plaque and gingival health were achieved at all test sites.

The descriptive statistics is presented in Table 1, which provides summary results and statistics for the measured variables. This includes the minimum and maximum values, the mean as a

Table 1. Descriptive statistics

	N	Minimum	Maximum	Mean	SD
serum LDH (U/l)	44	187.00	724.00	368.39	117.24
serum AST (U/l)	44	17.00	222.00	46.41	36.37
serum ALT (U/l)	44	13.00	366.00	71.86	69.30
GCF LDH (U)	44	4.00	59.00	43.16	14.15
GCF AST (U)	44	0.00	18.00	2.09	3.96
GCF ALT (U)	44	0.00	21.00	2.16	3.75

SD, standard deviation; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GCF, gingival crevicular fluid.

measure of central tendency and the standard deviation as a measure of dispersion.

The Kolmogorov–Smirnov Test comparing an observed cumulative distribution function to the theoretical normal cumulative distribution is presented in Table 2. The large significance values of serum LDH ($p = 0.142 > 0.05$) indicate that the observed distribution corresponds to the theoretical normal distribution. In contrast, the significance values for the rest of the variables are smaller than 0.05. The distribution is not normal for these variables and a non-parametric technique was used to further analyze them. The above results were verified by the histograms presented in Fig. 1.

The non-parametric Kendall's τ–b correlations table (Table 3) displays correlation coefficients and significance values. Kendall's τ–b was used to measure the strength of the association between variables measured at the interval level. The absolute value of the correlation coefficient indicates the strength, with larger absolute values indicating stronger relationships.

The significance of each correlation coefficient is also displayed in the correlation table. If the significance level was found less than 0.05, the correlation was considered significant and the two variables linearly related.

Table 2. Kolmogorov–Smirnov tests of normality

	Statistics	df	Significance
serum LDH	0.118	44	0.142
serum AST	0.216	44	**0.000**
serum ALT	0.198	44	**0.000**
GCF LDH	0.166	44	**0.004**
GCF AST	0.313	44	**0.000**
GCF ALT	0.283	44	**0.000**

LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GCF, gingival crevicular fluid. Bold numerals indicate not normal distribution for these variables.

The Kendall's τ–b correlation coefficient for serum LDH and serum AST was 0.252. The p-value was found to be $0.016 < 0.05$. The small significance level indicates that serum LDH and serum AST are significantly positively correlated. As serum LDH increases serum AST also increases (Fig. 2). Further, the Kendall's τ–b correlation coefficient for serum AST and serum ALT was 0.705 and the p-value was $0.000 < 0.05$. The small significance level indicates that serum AST and serum ALT is significantly positively correlated (Fig. 3).

Concerning the GCF measurements, the Kendall's τ–b correlation coefficient for GCF AST and GCF ALT was 0.705. The p-value is $0.000 < 0.05$. The small significance level indicates that GCF AST and GCF ALT are significantly positively correlated.

Discussion

One of the major goals of periodontal diagnosis is the identification of patients at high risk of destructive periodontal disease. It has been suggested that the nature of most forms of periodontitis is site-specific and that GCF protein composition analysis could be developed into a diagnostic tool for site-specific disease activity (McCulloch 1994). Several different methods have been used for the collection of GCF (Kavadia-Tsatala et al. 2002). The use of filter paper strips for the collection of native GCF is less disturbing to the crevicular epithelium and enables speedier measurements. Both of these advantages also further reduce the probability of altering the GCF by excessive contamination with serum (Insoft et al. 1996). The volume of GCF can be accurately determined between 0.2 and 0.4 μl with the Periotron®. However, the accuracy of this instrument below 0.1 μl is questionable (Insoft et al. 1996). As a result, small errors in volume determination can lead to large errors in estimating the final concentrations when the total volumes collected are small. According to Lamster et al. (1985a) and Insoft et al. (1996), measurement of the total quantity of mediator collected for a standardized short period of time will allow for more sensitive detection of site-to-site and patient-to-patient GCF differences without significant contamination with serum. Collecting GCF for long periods also increases the likelihood of saliva contamination.

LDH and AST have been used in assessing tissue destruction both in periodontal disease (Eley & Cox 1998) and thalassemia major (Hoffbrand et al. 1979, Wolfe et al. 1985, Triadou et al. 1989, Di Marco et al. 1993, Toren et al. 1996, De Simone et al. 1997).

It is well known that thalassemia major patients exhibit increased levels of the above enzymes in serum, which could possibly influence the GCF levels of the respected enzymes. The aim of this study was to determine any relationship between GCF and serum intracytoplasmic enzyme activity in periodontally healthy thalassemia major patients, and subsequently initially assess whether the increased serum levels of the aforementioned enzymes could possibly constitute a confounding factor in host-response-based tests used in periodontal diagnosis. Our results revealed that serum and GCF enzyme levels are not significantly positively correlated and therefore determining the association of GCF levels from periodontally affected sites and serum would be of interest as well. As the GCF is an osmotically mediated inflammatory exudates (Tollefsen & Saltvedt 1980, Cimasoni 1983), it is possible that with the greater GCF flow, the larger lesional volume and the increased permeability of the increased vascularity in the periodontal lesion GCF and enzyme levels correlations may be quite different.

LDH has earlier been correlated with clinical parameters in cross-sectional (Lamster et al. 1985b, c, Wolff et al. 1988), and longitudinal studies (Lamster et al. 1988, Wolff et al. 1988). However, LDH levels were not found to be predictive of disease activity (Kinane 1997, Eley & Cox 1998). Harper et al. (1989) reported a relationship between LDH and bacterial species found to be associated with chronic adult periodontitis. In a recent longitudinal study

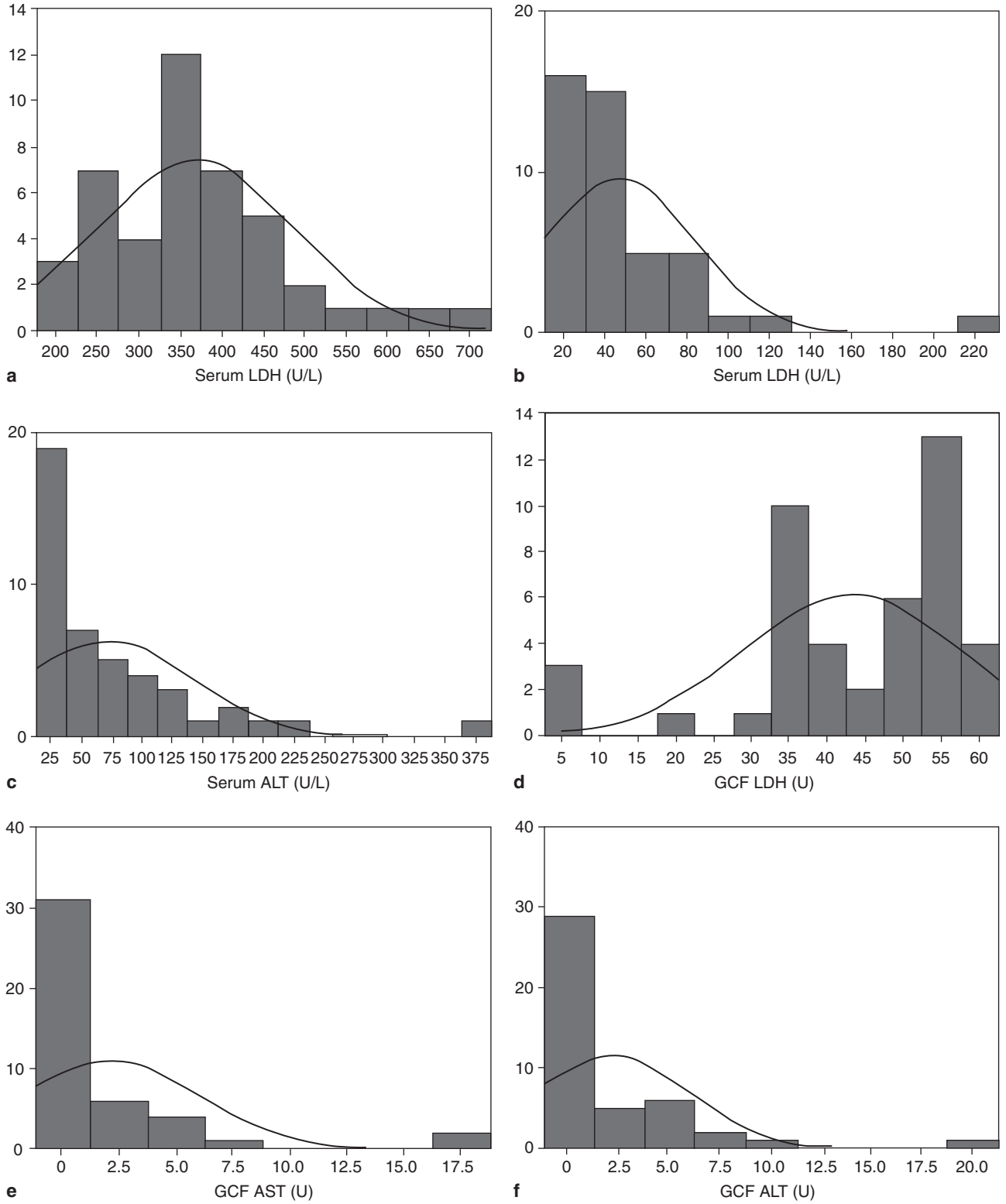

Fig. 1. Enzyme distribution in serum and gingival crevicular fluid.

enrolling adult and rapidly progressive periodontitis patients, Atici et al. (1998) observed baseline correlations between clinical parameters and LDH levels. However, they did not report any constant relationship throughout the experimental period, suggesting a lack of an absolute time-matching correlation between GCF profile and clinical periodontal status.

Chambers et al. (1984) were the first to demonstrate that AST levels in GCF increase during the development of periodontitis in beagle dogs. In experimental gingivitis in humans, GCF AST

Table 3. Kendall's τ–*b* correlations

	Serum LDH		Serum AST		Serum ALT		GCF LDH		GCF AST		GCF ALT	
	τ	*p*	τ	*p*	τ	*p*	τ	*p*	τ	*p*	τ	*p*
serum LDH	1.000	0.000	**0.252**	**0.016**	0.158	0.132	0.030	0.776	0.128	0.263	0.143	0.211
serum AST	**0.252**	**0.016**	1.000	0.000	**0.705**	**0.000**	− 0.079	0.459	0.073	0.529	0.128	0.265
serum ALT	0.158	0.132	**0.705**	**0.000**	1.000	0.000	− 0.013	0.903	0.050	0.662	0.038	0.742
GCF LDH	− 0.030	0.776	− 0.079	0.459	− 0.013	0.903	1.000	0.000	0.020	0.864	− 0.075	0.517
GCF AST	0.128	0.263	0.073	0.529	0.050	0.662	0.020	0.864	1.000	0.000	**0.711**	**0.000**
GCF ALT	0.143	0.211	0.128	0.265	0.038	0.742	− 0.075	0.517	**0.711**	**0.000**	1.000	0.000

LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GCF, gingival crevicular fluid. Bold numerals indicate a significant correlation between the variables.

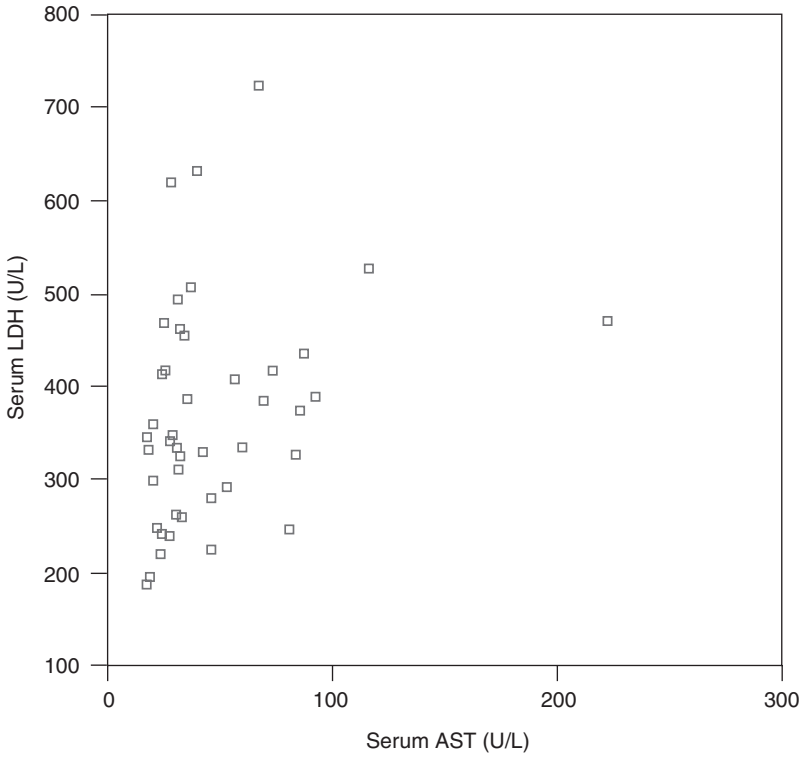

Fig. 2. Diagrammatic presentation of serum lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) values correlation.

samples were significantly associated with gingival inflammation during the various stages of development and resolution (Persson et al. 1990a). In cross-sectional studies, AST levels were reported to correlate with indices of disease severity at the patient level by Imrey et al. (1991) and they have a significant correlation with periodontally diseased sites. On the contrary, Shimada et al. (1999) failed to show a definite relationship between AST levels and individual measures of periodontal disease status. When the correlation of AST levels and microbiological features was examined, Kamma et al. (2001) and Kuru et al. (1999) in early onset periodontitis patients and Wong et al. (1999) in advanced periodontitis subjects, provided evidence of an association between putative periodontal pathogens and increased AST levels. In contrast, Smith et al. (1998) failed to demonstrate a positive association between AST levels with *Porphyromonas gingivalis* and *Actinobacillus. actinomycetemcomitans*.

In longitudinal studies, AST levels were shown to be strongly associated to confirmed disease-active sites, as assessed by attachment loss, in contrast to disease-inactive sites (Persson et al. 1990b, Chambers et al. 1991, Persson & Page 1992). GCF AST levels have also been recently shown to decrease after successful periodontal therapy (Atici et al. 1998, Shimada et al. 2000, Tsalikis et al. 2001). Persson et al. (1995) and Magnusson et al. (1996), using chairside test measuring GCF AST levels (PerioGard™, Xyntronyx, Inc., San Diego, CA, USA), concluded that diseased and non-diseased sites prior to and following therapy could be objectively distinguished in both patients and control subjects, and that therapy reduced test-positive sites. However, Oringer et al. (2001) produced contrasting evidence. The researchers used AST values recorded at baseline to assess the ability of the assay to identify disease progression over a 6-month period. Their results suggested that the AST assay was associated with a large number of false-positive results, but the high negative predictive value implies that a negative test result may be highly indicative of a periodontally stable site.

The current study involved 44 adults with β-thalassemia major. All patients received oral hygiene instructions, overhanging fillings and crown margins were polished, supra- and subgingival scaling and root planning were performed, prior to GCF and serum samples collection. Thus, they were considered to be periodontally healthy. Clinical parameters examination produced corroborating evidence.

Serum LDH and AST levels obtained from our sample of thalassemic patients were significantly higher than reported normal values (Bauer et al. 1974, Adolph & Lorenz 1982) and results produced by Atici et al. (1998) in a group of systemically and periodontally healthy subjects.

The lack of correlations between serum and GCF enzyme levels sug-

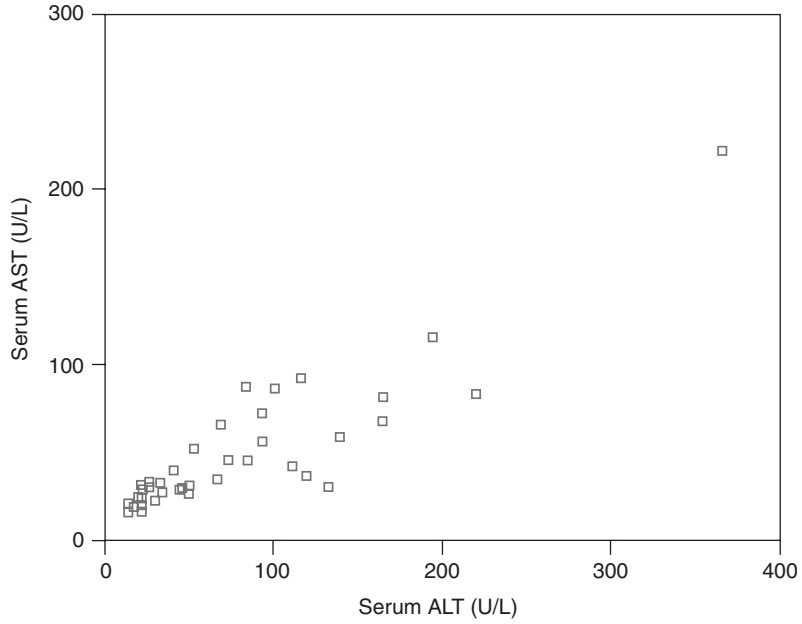

Fig. 3. Diagrammatic presentation of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values correlation.

gested that LDH and AST in serum do not constitute a confounding factor in GCF measurements of the respective enzymes. This is in accordance with previous studies suggesting the concept of local production of enzymes within the periodontal environment, rather than directly originating from blood cells or serum (Binder et al. 1987, Yamalik et al. 1990a, b, Atici et al. 1998), although those studies demonstrated markedly higher enzyme levels in GCF than serum. The significant correlation between AST and ALT levels both in GCF and serum suggests a common source of supply for these enzymes.

The present study, involving LDH, AST and ALT measurements both in GCF and serum, supports the validity of intracytoplasmic enzyme activity-based GCF analysis, for monitoring periodontal disease status in β-thalassemic patients.

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