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The Importance of Oral Mucosa and Minor Salivary Glands Biopsy for Diagnosing Chronic Graft-Versus-Host Disease. A Clinical Study of 188 Cases

SUMMARY

The biopsy examination of oral mucosa and minor salivary glands has been proposed as a valuable screening test for the diagnosis of chronic graft-versus-host disease (cGVHD). In this light, the purpose of our study was to illustrate the importance of biopsy in the early diagnosis of cGVHD. The study included 188 patients from 2003 to 2009, who had undergone allogenic bone marrow transplantation. The patients' ages ranged from 8 to 51 years. The distribution of the blood diseases was as follows: AA - 15; ALL - 46; AML - 70; CML - 19; MDS - 13; and NHL - 25. Approximately 3 months after the haematopoietic stem cell transplantation (HSCT), the patients were examined for the expression of cGVHD. A clinical diagnosis of "normal mucosa" was made for all the patients. The biopsy specimens were taken from the anterior left buccal mucosa, and from the minor salivary glands of the lower lip.

Of the 188 patients with no obvious clinical features of cGVHD, 108 patients (57.44%) showed positive expression of cGVHD in the histological examination, whereas 80 patients (42.53%) showed negative expression of cGVHD. The statistical analysis revealed a higher occurrence of cGVHD (p=0.041) in younger patients of those with AML and CML, whereas among patients with ALL and NHL, the older ones were diagnosed more frequently with cGVHD (p=0.04).

Keywords: Graft-Versus-Host Disease; Oral Mucosa; Minor Salivary Glands

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Introduction

The growing success of allogenic haematopoietic stem cell transplantation (HSCT) in the treatment of malignant haematological diseases has contributed to a steady increase in its use[1-5]. In HSCT, the primary disease is eradicated according to the established protocols, by use of chemotherapy or radiation therapy, and then the bone marrow is replaced by harvested donor haematopoietic stem cells[6].

Graft-versus-host disease (GVHD) is one of the most important complications of HSCT and is a leading cause of morbidity and mortality in HSCT patients. GVHD develops when the transplanted donor immune cells react and try to destroy the host tissues[5]. The pathophysiology of GVHD consists of 3 stages: stage I is characterized by tissue damage caused to the recipient; stage II is related to the activation of the donor T-cells which recognize the host antigens as foreign and attack target organs; and, finally, stage III involves the release of cellular and inflammatory factors, such as cytokines, endotoxins, and other bacterial products[6,7].

Depending on the time of disease onset, GVHD is divided into acute GVHD (aGVHD), in which clinical features appear within 100 days after bone marrow transplantation, and chronic GVHD (cGVHD), if clinical features occur later[5,6,8]. Nowadays, with the advances in HSCT, criteria for acute and chronic GVHD have changed, and the current belief is that the distinction between aGVHD and cGVHD is made on the basis of

clinical features rather than the time of symptoms onset after transplantation[5,6]. Clinical picture in acute GVHD comprises an erythematous rash, diarrhea, and/or liver involvement. Chronic GVHD is a distinct syndrome which can affect every major organ system, but most commonly involves skin, oral, vaginal and conjunctival mucosa, salivary and lacrimal glands, as well as the liver[6,9].

One of the main barriers to the conduct of effective clinical research in cGVHD has been the absence of standardized criteria for the diagnosis and staging of the disease, as well as the response to therapy. The National Institutes of Health (NIH) Consensus Development Project on Criteria for clinical trials in cGVHD has recently proposed standardized criteria for the diagnosis of cGVHD. The working group recommended that the diagnosis of cGVHD should require at least 1 diagnostic manifestation of cGVHD, or at least 1 distinctive manifestation (Tab. 1), with the diagnosis being confirmed by a pertinent biopsy, laboratory tests, or radiology in other organs[10]. Because the NIH gave the most recent system of clinical criteria for cGVHD, few studies using it have been published in the English literature.

Table 1. Criteria of cGVHD with emphasis on lesions in oral mucosa

Diagnostic (sufficient to establish the diagnosis of cGVHD	Distinctive (seen in cGVHD, but insufficient alone to establish a diagnosis of cGVHD	Common (seen with both aGVHD and cGVHD
Lichen-type features Hyperkeratotic plaques Restriction of mouth openings from sclerosis.	Xerostomia Mucocele Mucosal atrophy Pseudomembranes Ulcers	Gingivitis Mucositis Erythema Pain

A.H. Filipovich et al 2005

Even though cGVHD can affect every organ system, the most commonly involved are skin and oral mucosa[6,11]. Oral involvement occurs in 80% or more of cGVHD patients, and the typical clinical features include lichenoid changes (white reticulation and/or plaques), ulcerations (yellow-to-white pseudomembranes), mucosal atrophy, salivary gland dysfunction (xerostomia, hyposalivation), and restricted oral opening. Mucosal lesions are similar to those encountered in oral lichen planus, salivary gland infiltrates mimic those found in Sjögren syndrome, while fibrosis and restricted oral range of motion suggest scleroderma. Common clinical oral signs of both aGVHD and cGVHD include mucositis, gingivitis, oral erythema, and pain[5,10-12].

Although xerostomia is a commonly reported complaint in cGVHD, criteria for evaluation of the prevalence and characteristics of salivary gland involvement have not been well-defined in the literature because complaints of oral dryness are regarded as “oral” or “mouth” involvement, whereas pathologic changes in the minor salivary glands encountered in cGVHD are seen as a continuation of oral mucosal lesions found in the disease. It is also remarkable that, in recent NIH reports, criteria for salivary and mucosal involvement were grouped together as “oral involvement”[10,13-15].

Oral clinical examination and the biopsy of oral mucosa and minor salivary glands have been proposed as valuable screening tests for the diagnosis of cGVHD about 3 months following transplantation, due to the high incidence of oral mucosa involvement, and high predictive value of the examination (nearly 100%)[8]. It is believed that an oral cGVHD lesion may be the only observed indicator of cGVHD, systemic cGVHD being defined by that.

The purpose of this study was to provide more knowledge on cGVHD, particularly when the disease occurs in patients with no clinical features in the oral cavity and other systems (skin, liver, gastrointestinal tract), showing the importance of biopsy of the oral mucosa and minor salivary glands for early diagnosis of the disease.

Materials and Methods

In the period between January 2003 and December 2009, a total of 835 patients were referred to our clinic at the “G. Papanikolaou” Hospital in Thessaloniki by the Haematological Clinic for diagnosing cGVHD by use of biopsy of oral mucosa and minor salivary glands. All these patients had undergone allogenic HSCT for haematological malignancies. From this large number of patients, we excluded those with obvious oral clinical findings (lichenoid lesions, erythema, xerostomia, etc). From the remaining patients, we selected a group of 188 patients who showed normal oral mucosa and no involvement of other systems (skin, liver or gastrointestinal tract). This final group of patients was studied for cGVHD under similar clinical parameters. No

discrimination was made about the patients' nationality, social status, gender or age.

There were 110 males and 78 females. The patients' ages ranged from 8 to 51 years. The distribution of the blood disease was as follows: aplastic anaemia (AA) - 15; acute lymphocytic leukaemia (ALL) - 46; acute myeloid leukaemia (AML) - 70; chronic myeloid leukaemia (CML) - 19; myelodysplastic syndrome (MDS) - 13; and non Hodgkin lymphoma (NHL) - 25. All the patients had undergone allogenic bone marrow transplantation (BMT).

About 3 months (100 days) after the HSCT, the patients were examined for the presence of oral mucosal lesions or gingival diseases. A clinical diagnosis of "normal mucosa" was made for all the patients based on their medical history and a thorough clinical examination. As "normal" we regarded mucosa that had no oral lesions (lichenoid hyperkeratotic lesions), no infection (gingivitis, mucositis, erythema) or xerostomia, no signs of hyposalivation (salivary flow rate at 0.2 ml/min or 1ml/5min unstimulated), no minor salivary gland mucoceles, oral mucosa atrophy, oral pseudomembranes or ulcers. None of the 188 patients showed any underlying infection, any oral malignancies and received only prophylactic treatment (no other drugs as steroids etc). Other systems, such as the skin, liver, and gastrointestinal tract, were not involved.

The diagnosis of cGVHD was established by biopsy of the oral mucosa and minor salivary glands. Biopsies were performed approximately 100 days after the BMT, under local anesthesia. 1 biopsy was taken from 2 sites in all 188 patients - the oral mucosa (anterior left buccal mucosa) and the minor salivary glands (lower lip). Histological specimens were fixed in formalin and embedded in paraffin. H & E stained sections were evaluated by a pathologist with expertise in cGVHD, who was blinded to the clinical evaluation results. In the specimens of oral mucosa a presence of epithelial atrophy with apoptotic bodies, hydropic degeneration of basal cells, interface mucositis, or subepithelial lymphocyte infiltration of the connective tissue was evaluated. Also, in the specimens of minor salivary glands a presence of diffuse/periductal lymphocyte infiltrate, atrophy or destruction of acini, ductal dilatation or fibrosis was examined.

Statistical Analysis

Data were analyzed with the SPSS program for Windows (version 10.0, Chicago, IL, USA). The Kolmogorov-Smirnov and the Shapiro-Wilk tests were used to check normality of continuous variables. Results are presented as mean ± standard deviation (SD) for parametric variables and median (range) for non-parametric variables. The ANOVA, Kruskall-Wallis and Mann-Whitney tests were used to calculate the significance of differences. Qualitative data were also examined by use of the χ^2 or Fisher exact test. The age variable was used either quantitatively or qualitatively. Statistical significance was defined as $p \leq 0.05$.

Results

The mean age of the investigated patients was 31.78 years (SD = 10.86 years). Of the 188 patients with no obvious oral clinical features of cGVHD, 108 patients (57.44%) showed positive expression of cGVHD, whereas 80 patients (42.53%) showed negative expression of cGVHD on histological examination (Tab. 2).

Table 2. Expression of cGVHD

Blood disease	cGVHD (+)	cGVHD (-)
AA (Aplastic anaemia)	6	9
ALL (Acute lymphocytic leukaemia)	24	22
AML (Acute myeloid leukaemia)	44	26
CML (Chronic myeloid leukaemia)	14	5
MDS (Myelodysplastic syndrome)	6	7
NHL (Non Hodgkin lymphoma)	14	11
	108	80

All the histological findings represented minimal histological criteria of cGVHD in this study with different degree of expression. The most frequent findings in oral mucosa were localized or generalized epithelial changes consisting of lichenoid interface inflammation, hydropic degeneration of basal cells, or interspersed areas of atrophy with apoptotic bodies. In the connective tissue, variable amounts of perivascular inflammation and lymhocytic infiltration could be found (Fig. 1). Minor salivary glands revealed periductal infiltration with lymphocytes, atrophy of salivary gland lobules and periglandular fibrosis (Fig. 2).

The distribution of the patients according to blood disease and with regard to gender and age is presented in Table 3. Table 4 presents all statistically significant correlations between the examined parameters (blood disease, age, cGVHD). No significant overall statistical correlation was observed between gender and blood disease (df=5; p=0.166), gender and cGVHD (df=1; p=0.295), age and gender (f=0.077; p=0.782), and age and cGVHD (f=0.757; p=0.385).

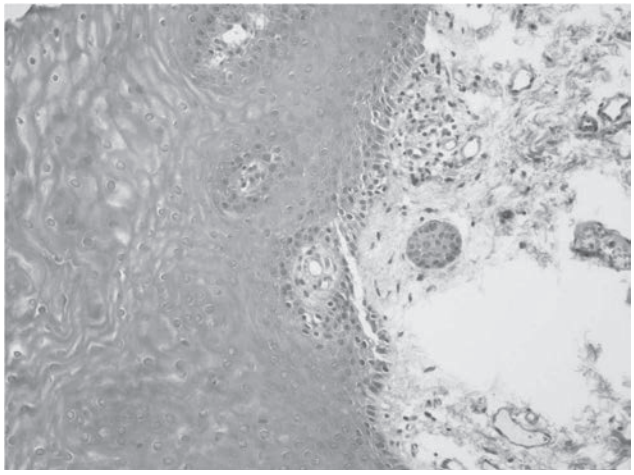

Figure 1a. Normal histology of oral mucosa (HEx200)

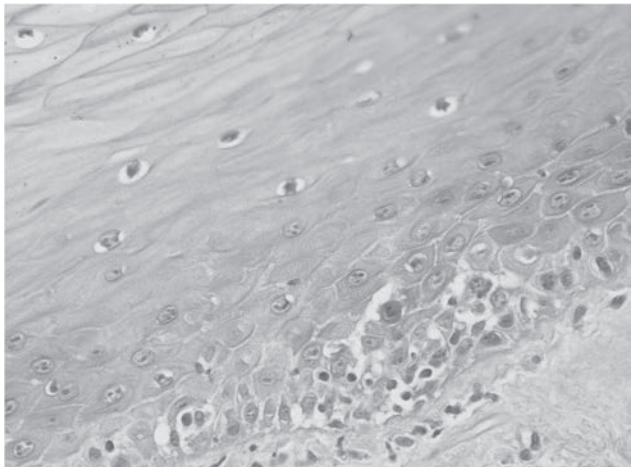

Figure 1b. Chronic GVHD of oral mucosa - lymphoplasmatic infiltration of the upper lamina propria with focal invasion of basal epithelial layers, and single apoptotic body (HEx400)

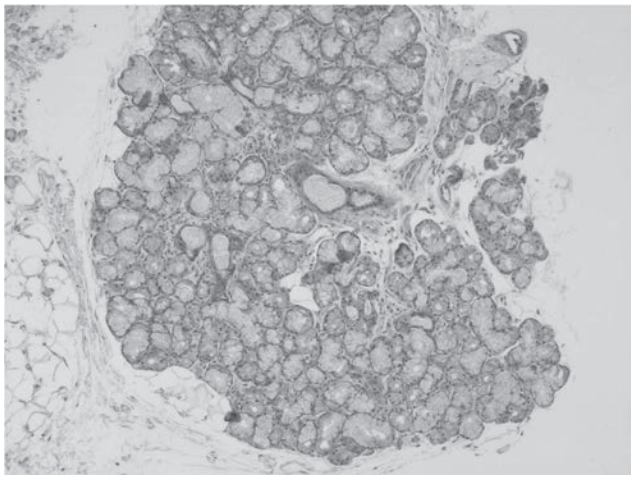

Figure 2a. Normal histology of minor salivary gland (HEx200)

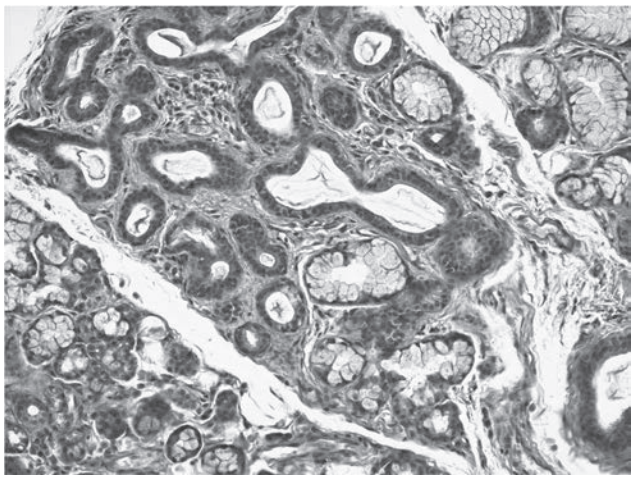

Figure 2b. Minor salivary gland tissue shows acinar atrophy and destruction, as well as an extensive periductal fibrosis (HEx200)

Table 3. Distribution of patients according to blood disease, gender and age

				Age			
Disease	N	% of total	M/F	Mean	SD	Min	Max
AA	15	8	8/7	19,40	7,44	10	35
ALL	46	24.5	25/21	27,46	10,09	8	50
AML	70	37.2	38/32	34,86	9,33	8	51
CML	19	10.1	11/8	34,84	10,13	16	51
MDS	13	6.9	7/6	42,62	7,16	31	51
NHL	25	13.3	21/4	30,56	10,57	16	51
Total	188	100	188	31,78	10,86	8	51

Table 4. Statistical correlations between examined parameters (blood disease, age, cGVHD)

			p GVHD	disease	(Y/O)	Diagnosis
AA	ALL	Age	0,04	0,007	Younger	AA
	AML	Age	ns	<0,001	Younger	AA
	CML	Age	ns	<0,001	Younger	AA
	MDS	Age	ns	<0,001	Younger	AA
	NHL	Age	ns	0,001	Younger	AA
ALL	AML	Age	ns	<0,001	Younger	ALL
	CML	Age	ns	0,01	Younger	ALL
	MDS	Age	ns	<0,001	Younger	ALL
	NHL	Gender	0,046	0,018		
AML	CML	Age	0,041	ns		
	MDS	Age	ns	0,006	Younger	AML
	NHL	Gender	ns	0,009		
CML	MDS	Age	ns	0,024	Younger	CML
MDS	NHL	Age	ns	0,001	Oldest	MDS

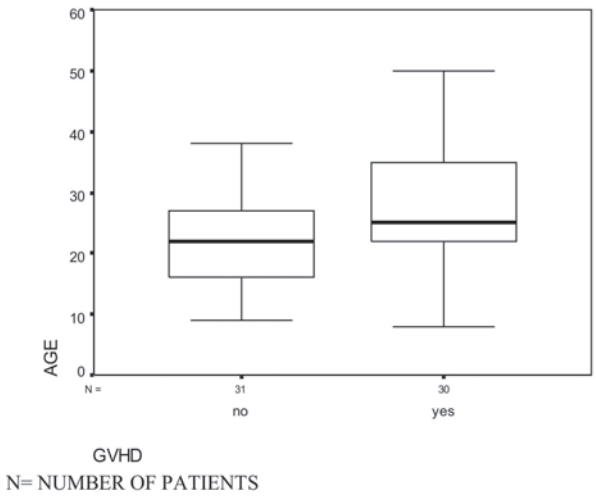

Figure 3. AA-ALL expression of cGVHD in older patients

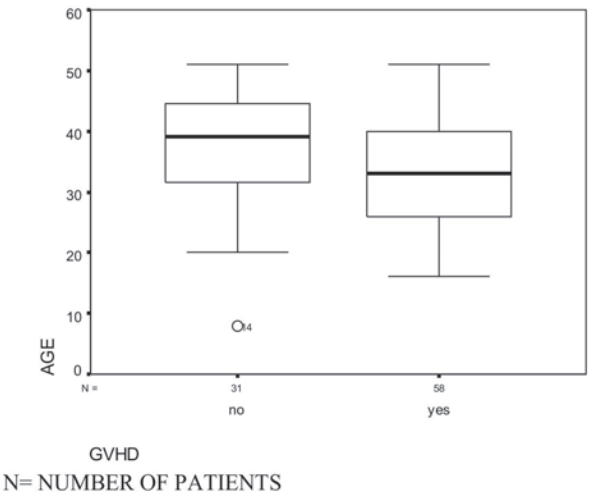

Figure 4. AML-CML expression of cGVHD in younger patients

Among patients with AA and ALL (Fig. 3), the older patients were diagnosed more frequently with cGVHD (p=0.04). Regarding patients with AML and CML (Fig. 4), the younger developed higher rates of cGVHD (p=0.041). Among patients with ALL and NHL, the male patients were diagnosed more often with cGVHD (p=0.046).

Discussion

Chronic GVHD remains the most significant long-term challenge after allogenic HSCT. It is estimated that 40% to 70% of transplanted patients surviving the initial transplantation will eventually develop cGVHD that

requires long-term treatment[5,16]. Therefore, earlier and more precise diagnosis is important both for the timely application of an effective therapeutic schema, as well as for a successful long-term follow-up of the malignant disease. As mentioned earlier, clinical features of oral mucosa and minor salivary glands have been noted to reflect the development of cGVHD better than any other affected organs. Minor salivary glands can be affected by cGVHD more frequently than oral mucosa, probably due to the higher tissue expression of histocompatibility antigens by salivary tissues and the accessibility of glands to pathogenic lymphocytes[5,8].

As mentioned in the literature, histological features of oral cGVHD resemble those of oral lichen planus to a certain degree, but the inflammatory response in cGVHD is usually not as intense as in lichen planus[10]. Minimal histological criteria for oral mucosal cGVHD are localized and extensive epithelial changes (lichenoid interface inflammation, exocytosis and apoptosis), whereas the connective tissue is characterized by variable amounts of perivascular inflammation and lymphocytic infiltration[5,8,17-19]. Concerning minor salivary glands, histological criteria characteristic for cGVHD are lymphocytic infiltration of salivary gland ducts, individual ductal epithelial cell necrosis (apoptosis) and destruction of acinar tissues with periductal fibrosis[8,19]. Obviously, the results of our study are in compliance with those in the literature.

The biopsy as a means of confirming clinical diagnosis of current cGVHD is recommended in the cases when an alternative diagnosis is possible, when there are no diagnostic clinical features of cGVHD, or when only internal organs exhibit clinical signs of current cGVHD. In all these implications and also when infections with atypical clinical features are present, a biopsy is essential to establish a correct diagnosis of cGVHD[20]. Though controversial, the evaluation of biopsy specimens is also a challenging issue in distinguishing the current disease from the past disease. Dense fibrosis and acinar destruction most probably reflect past disease, while acinar and periductal inflammation most probably reflects current cGVHD. As is believed, such histological implications relate to patients who have undergone more than one transplantations[5,7,8].

Several clinical studies have reported that oral lesions are common in patients with cGVHD, which is estimated to occur in 45% to 83% of the patients[21,22]. Flowers et al[1] noted that oral mucosa was the second most common affected site in those patients who developed cGVHD. Mohty et al[23], in their study of 101 patients, showed that 51% of the surviving patients developed oral cGVHD during the first 3 years after transplantation. Similar 3-year cumulative incidence of cGVHD with oral lesions was found in the cohort of 126 patients monitored by Flowers et al[1] with just under 50% of the patients developing oral cGVHD during the first 3 years. Treister et al[21] reported oral features in 49 children who had been transplanted for a variety of benign and malignant diseases.

Approximately 90% of the patients had a history of any cGVHD, and about 50% were found to have oral cGVHD (erythema, atrophic glossitis, superficial mucoceles, etc.). Hiroki et al[24] examined 14 patients who received allogenic BMT. 10 of 14 patients were diagnosed as having cGVHD in skin, liver, and other organs. The cGVHD patients had also objective evidence of oral involvement (xerostomia, lichenoid lesions). The conclusion of their study was that oral examination, including biopsy of oral mucosa, is useful for the diagnosis of cGVHD. The same authors[25], 2 years later, examined 37 patients who had undergone an allogenic BMT and compared oral findings with systemic involvement of cGVHD. The results of their study suggested that a systematic oral examination, especially pathologic examination of the labial salivary gland and buccal mucosa, is useful in evaluating the status of cGVHD. Resende et al[26] selected 60 patients with diagnosed systemic cGVHD and studied the relationship between systemic cGVHD and the oral lesions. Although their results concerning the accuracy of oral cGVHD tests was low for the diagnosis of cGVHD, the conclusion of their study was that the presence of oral symptoms and histopathological manifestations in the salivary glands have good properties for the diagnosis of cGVHD.

Although there are clinical studies on the appearance of cGVHD in transplanted patients with obvious clinical features in the oral cavity, there are no clinical studies about the appearance of cGVHD in transplanted patients with no oral clinical features or oral lesions. Demarosi et al[6] studied the cGVHD in 13 patients who had been transplanted for haematological malignancies. Biopsy specimens were taken from 4 patients with clinical manifestations of oral cGVHD and from 9 patients with normal oral mucosa. Histological cGVHD changes were detected in each one of the 4 patients (100%) with clinical manifestations of oral cGVHD and in 6 of the 9 patients (66.6%) with apparently healthy oral mucosa. The same authors mentioned that the number of patients was insufficient for a definite diagnosis of oral cGVHD with no oral clinical features. Nevertheless, a longer follow-up period in patients showing histological changes of cGVHD with no clinical features may be useful for the further development of the disease.

In our study, we studied 188 patients without any oral clinical feature of cGVHD, with 108 of these patients having been found positive for cGVHD. Even though these histological features in the oral mucosa without corresponding clinical symptoms may be considered insufficient for a definite diagnosis of cGVHD, this status, which is recognized by some authors as subclinical cGVHD, may be the only highly predictive index of the presence of cGVHD, or a sign of future possible outbreak

of the disease. The investigated parameters revealed significant correlations between the blood disease (AA-ALL and AML-CML), the age of the patients, and the expression of cGVHD. The conclusions are analyzed in figure 1 and figure 2, and it is obvious that older patients with AA and ALL were more frequently diagnosed with positive expression of cGVHD, while among patients with AML and CML, younger ones were diagnosed with positive expression of cGVHD more frequently. It has been reported that the incidence of cGVHD in patients who survived after HSCT is as follows: 13% in patients younger than 10 years, 28% in patients aged 10 to 19 years, and over 40% in patients older than 20 years. However, no correlation is made between the blood disease and the age of the patients[27].

Pathophysiology of cGVHD remains indefinite and progress in the development of effective therapeutic and preventive schemata proceeds very slowly. Moreover, there are close clinical and histopathological similarities between cGVHD and autoimmune disorders. Therefore, progress in cGVHD research may be beneficial not only to HSCT patients, but also to larger number of patients[5].

Biopsy of oral mucosa and minor salivary glands is an easy surgical procedure and we believe, according to the results of our study, that it can contribute significantly to the diagnosis of cGVHD, even when oral clinical features are absent. The histological findings of the biopsy examination are evaluated by haematologists who monitor the development of the disease in the course of time. Some of these patients, even without clinical evidence, will develop cGVHD, to which a better therapeutic approach will be possible thanks to early diagnosis. This is what constitutes the importance of the biopsy of oral mucosa and minor salivary glands, and it is why it has been established in literature as a diagnostic criterion.

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