**Conjunctival biopsy site in mucous membrane pemphigoid**

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**Short title:** mucous membrane pemphigoid and conjunctival biopsy

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**Introduction**

Mucous membrane pemphigoid (MMP) is a rare systemic immune-mediated disease that can affect several mucous membranes including the oral cavity, eye, nose, respiratory tract and gastrointestinal tract. Ocular involvement presents as a chronic, relapsing-remitting bilateral cicatrizing conjunctivitis with progressive conjunctiva fibrosis, symblepharon formation, loss of the fornices and entropion. This may lead to corneal ulceration and opacification with loss of vision that can cause bilateral blindness in 20% of cases. 1234 MMP is characterized by the presence of autoantibodies which recognize and react against antigens expressed at the basement membrane zone (BMZ) in the epithelial-sub epithelial junction of mucous membranes 5 and this autoimmune reaction leads to inflammation and subsequent scarring of the involved areas.

The diagnosis of MMP involves a combination of clinical, histological and immunopathological features. 6 The first international consensus on MMP concluded that both clinical findings and direct immunopathology results are essential to establish a diagnosis of MMP. 6 A direct immunofluorescence (DIF) test is considered the gold standard for the diagnosis of MMP. 6 DIF is able to detect deposition of immunoglobulins (IgG, IgA, and/or IgM) and/or complement (C3) in the epithelial BMZ. 6 It has a reported sensitivity of 70-80%. 7891011 Although repeated and the simultaneous sampling of several sites has been advocated to increase the diagnostic sensitivity of DIF 1213; it would be preferable to avoid repeated biopsies of the conjunctiva and to therefore increase the yield of the initial biopsy.

Although it is well established that in cases of oral and/or skin involvement, biopsies should be taken from perilesional sites 6, it is not known whether the same applies in cases of ocular involvement. In addition, it is not clear which area of the ocular surface should be sampled particularly as sensitivity of DIF test performed in conjunctival biopsies can be as low as 30%. 14 It has been suggested that this reflects differences in the reactivity of conjunctival samples to immunofluorescence testing. 15 The purpose of our study was to evaluate if there was an association between the location of the conjunctival biopsy (lesional, peri-lesional or non-affected) and the DIF result.

**Methods**

We conducted a retrospective analysis of patients with clinically suspected MMP who underwent conjunctival biopsy from March 2014 to February 2019 at The Royal Liverpool University Hospital, United Kingdom. Patients were considered to have suspect MMP based on clinical judgment, when various combinations of conjunctival fibrosis, fornices shortening, conjunctival cicatrization, symblepharon, corneal neovascularization, corneal scarring, cicatricial blepharitis, entropion and trichiasis were present. A note was also made of patients, who had reported oral signs and/or symptoms and were assessed in the department of Oral Medicine, Liverpool University Dental Hospital. The ocular surface location of the conjunctival biopsy was reported in the patients’ electronic charts. Location of the conjunctival samples was defined as 'lesional' when the sample was taken from a well-defined area of conjunctival scarring or symblepharon, 'peri-lesional' when the sample was taken from an area of clinically uninvolved conjunctiva adjacent to a lesion and 'non-affected', when the sample was taken from a non-affected distant area, either from forniceal conjuctiva or bulbar conjunctiva. Conjunctival biopsies were performed in the operating theatre under local anaesthesia (topical minims proxymetacaine hydrochloride 0.5% w/v, eye drops solution, and subconjunctival lidocaine 2%). The size of conjunctival biopsy was not specified in patient’s charts. Samples were placed in Michel’s transport medium and sent to the St. John’s Institute of Dermatology in London for further analysis. All conjunctival samples were analysed using direct immunofluorescence to detect the deposition of IgM, IgG, IgA, and C3 at the BMZ. 12 Results of the direct immunofluorescence analysis were labelled as 'positive', when there was deposition of at least one of either IgM, IgG, IgA, and C3 at the basement membrane of the specimen, 'non-specific' when only fibrinogen was found at the same location and 'negative' when none of these features were present. Positive results were also considered to be diagnostic, while both non-specific and negative results were non-diagnostic. The study was approved by the Institutional Review Board (IRB). Statistical analysis was performed using the Fisher’s exact test to detect a difference between diagnostic (positive) and non-diagnostic (non-specific and negative) results according to sample location (lesional, peri-lesional and non-affected conjunctiva). Data are presented as mean ± standard deviation (SD). A p-value of < 0.05 was considered statistically significant. Bonferroni correction for multiple comparisons was applied when appropriate.

**Results**

The records of forty-one patients who underwent conjunctival biopsy for suspect MMP were available for analysis. Mean age at the time of the biopsy was 74±12.1 years and 27 were females (66%). Of the forty-one patients, nine were excluded from the study because no information was available on the location of the biopsy or because no epithelium was present in the specimen. A total of 32 patients were included in the analysis. The 32 patients analysed (22 females) had a mean age of 72±12 years. Nine patients also had oral symptoms of whom 6 also had oral DIF biopsies. The demographics and baseline clinical details of the study population are shown in Table 1. Biopsies were lesional in 22% of cases (7/32), peri-lesional in 22% (7/32) and from non-affected conjunctiva in 56% (18/32). Of these, nineteen percent were from the bulbar conjunctiva and 37% from the inferior forniceal conjunctiva. Overall, 10/32 (31%) biopsies gave a positive DIF test (3 of these patients also had an oral biopsy which gave a positive DIF result in one case and negative DIF result in the remaining two. Thirteen of the 32 patients (41%) had a non-specific DIF result and 9/32 (28%) a negative DIF result. Of these 22 patients 3 also had an oral biopsy and these all gave a negative DIF result.

Results from lesional biopsies were positive in 14% of cases (1/7), non-specific in 57% (4/7) and negative in 29% (2/7). Peri-lesional biopsies were positive in 86% of cases (6/7), non-specific in 14% (1/7) and none were negative. Biopsies taken from areas of non-affected conjunctiva were positive in 17% (3/18) (17% for bulbar conjunctiva and 17% from forniceal conjunctiva), non-specific in 44% (8/18) (50% of bulbar conjunctival samples and 42% of forniceal conjunctival samples) and negative in 39% (7/18) (33% and 42% for the bulbar and forniceal conjunctiva respectively).

There was a significant difference in DIF from samples taken from lesional, peri-lesional and non-affected conjunctiva (p=0.003) (Table 2). DIF from peri-lesional biopsies was more informative than from lesional sites (p=0.029) (Table 2).

After a mean follow-up of 1.93±1.4 years six patients were diagnosed as non-MMP, while the remaining 26 patients were still considered to have MMP. The diagnosis of non-MMP was made when an alternative cause of cicatricial conjunctivitis was considered to potentially explain the clinical ocular findings, while patients in whom no alternative diagnosis could be made, were still considered to be affected by MMP and these latter cases were defined as either definite MMP, if the DIF test confirmed the diagnosis, or suspected MMP if DIF result was negative. One patients in the suspect MMP group had lichen planus of the mouth and one patient had Bowen’s disease of the skin. Analysis in this group of patients showed that overall, 10/26 biopsies were positive (38.5%), 10/26 non-specific (38.5%) and 6/26 negative (23%). Lesional biopsies were positive in 1/6 (17%) and either non-specific (3/6) or negative (2/6) in 5/6 (83%). Peri-lesional biopsies were positive in 6/6 (100%) of cases and biopsies from non-affected conjunctiva were positive in 3/14 (21.5%) and either non-specific (7/14) or negative (4/7) in 11/14 (78.5%) (p=0.002) (Table 2 and Figure 1).

**Discussion**

We investigated the association between the site of conjunctival biopsy and the DIF result in patients with clinically suspected MMP. Although there is good evidence of the need for peri-lesional biopsy in patients with MMP with oral and/or skin involvement, this has not been investigated in biopsies from the conjunctiva of patients with ocular involvement. 6 Results from our study support the need to sample peri-lesional conjunctival tissue in patients with suspected MMP in order to increase the sensitivity of DIF on conjunctival samples. The current diagnosis of MMP is based on the first international consensus criteria according to which, both clinical findings and direct immunopathology results are essential to establish the diagnosis 6. Although it is recognised that DIF has limited sensitivity and specificity in MMP 46, it provides useful diagnostic information to aid an appropriate management plan. Oral mucosa and skin biopsies have been shown to have on average 70-80% DIF positive results. 91016 In contrast, results from conjunctival samples are lower compared to other sites and also vary widely both within and between studies, from 30-80%. 17121418 Since it is well accepted that extraocular sites biopsies have higher sensitivity to detect the disease, in cases of MMP with extra-ocular involvement, biopsies should be taken from a non-ocular site. 6 When there are only ocular manifestations, it has been suggested that the diagnosis of MMP should be made, even in the absence of a positive DIF result, when clinical signs are strongly suggestive of the disease and other causes of cicatricial conjunctivitis have been excluded. 41920

It has been suggested that one of the reasons why the DIF test sensitivity is lower in conjunctival tissue, is that the conjunctiva might react differently with DIF. 15 We would suggest that this low rate of positive results may reflect a mixture of conjunctival samples taken from different ocular surface locations. In addition, inflammatory non-specific results, such as the presence of a fibrinogenous band at the basement membrane zone, have been reported with higher frequencies in conjunctival samples and have also been reported to be a normal finding in conjunctival specimens 21. The highest percentage of DIF positive results in conjunctival samples have been reported by Thorne et al., in a large retrospective study conducted in 2004. They found a positive DIF result of approximately 80%, collecting the specimen from the inferior forniceal conjunctiva of an affected eye but, unfortunately, they did not specify whether they were taken from lesional, peri-lesional or non-affected areas. 12

Although simultaneous multiple biopsies are suggested to increase the sensitivity of DIF results 13, a recent study by Shimanovich et al., showed that more than 30% of simultaneous multiple biopsies presented with discordant results. This means that different areas collected from the same patient and at the same time reacted differently using DIF. It can be argued that these findings were due to poor biopsy site selection, either too close or too far from a lesional area or due to an uneven distribution of mucosal autoantibodies. 13 The inflammatory process in lesional areas might alter the immuno-reactants and although it is considered that autoantibodies potentially involve the entire bodies mucosa 11, they they may be absent at distant sites. 13 DIF results might be more variable on the basis of sample location than previously thought and a single negative DIF results cannot exclude MMP. 13 Whilst repeated sampling, has therefore been advocated 1213 it would be preferable to avoid repeated conjunctival injury and maximise the yield of the biopsy.

In our study, DIF sensitivity was 31%, which is on the low side of that reported in the literature. This, however, increased to 38% after having excluded patients who were revealed not to have MMP on the follow-up. One of the explanations for our percentage might be that most of our conjunctival biopsies were taken from non-affected areas, thus probably including areas with absence of autoantibodies. We acknowledge that one of the limitations of our study is that it is a retrospective analysis, has a small sample size and we cannot exclude that more MMP patients were in the group which received peri-lesional biopsies by chance. Nevertheless, at the time of biopsy all patients had the same pre-test probability of being positive to the DIF test, which would support the findings. Thus far, only one study has compared the DIF result with the biopsy site in ocular MMP. Mehra et al., reported that while there were no differences in the presence of IgA, IgM, C3 and fibrinogen between lesional and non-lesional conjunctival biopsies, the sensitivity of IgG, which is the most common finding in MMP222324, was double in non-lesional samples compared to lesional ones, reaching values of around 40% 15. Although they did not compare peri-lesional and lesional and non-lesional sites, this does point to the importance of the biopsy site in determining the DIF test result 15. Ocular MMP is a systemic disease, and extraocular involvement can affect more than 80% of cases at the time of diagnosis 12. There is multiple evidence to support the need for systemic immunosuppression treatment 25262728 in patients with MMP and therefore, DIF results have important clinical implications in patient management 20. Labowsky et al., reported that patients with negative DIF results were less likely to receive systemic immunosuppressive drug therapy and had on average shorter follow-up time. 20 This is an important observation that underlies the need to improve detection of a positive DIF result and thus provide optimal care for these patients. In conclusion we found that peri-lesional conjunctival biopsies in ocular MMP patients increase the sensitivity of DIF testing. The findings of our study would support a recommendation that, in patients with clinically suspected MMP, biopsies should be taken from peri-lesional sites.

**Acknowledgement Section**

A. Funding/Support: None

B. Financial Disclosures:  No financial disclosures

C. Other Acknowledgments: None

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Table 1. Demographics and clinical details of the study population at baseline.

Table 2. Data on DIF results detailed for biopsy site and biopsy result. The top half of the table refers to the whole study population (n=32) and the bottom half refers to the analysis (n=26) of patient who were still considered to be affected by MMP after follow-up, either definite or suspect MMP. Data are presented as proportions and percentages. Fisher’s exact test p-values are shown. DIF: direct immunofluorescence; MMP: mucous membrane pemphigoid.

Figure 1. Graphs showing percentages of diagnostic (positive) Vs non-diagnostic (non-specific and negative) results in lesional, peri-lesional and non-affected conjunctival biopsies. **A.** Results refer to the whole study population analysed (n=32); **B.** Results referring to the analysis of patient who were still considered to be affected by MMP after a mean follow up of 1.93±1.4 years (n=26). Fisher’s exact test p-values are shown in the graph. Bonferroni corrected p-values for multiple comparisons when appropriate.