

## Post-transplant events

# The relationship between mouthrinse matrix metalloproteinases (MMP-1, 8, 13) and albumin levels with the degree of oral mucositis in allogeneic stem cell transplant patients

I Shoval<sup>1</sup>, JA Kushner<sup>1</sup>, B Sukhu<sup>2</sup>, R Wood<sup>1</sup>, T Kiss<sup>3</sup>, HP Lawrence<sup>1</sup> and HC Tenenbaum<sup>1,4</sup>

<sup>1</sup>Faculty of Dentistry, University of Toronto, Toronto, Canada; <sup>2</sup>Department of Pathology, Mount Sinai Hospital, Toronto, Canada; <sup>3</sup>Department of Hematology, Maisonneuve Rosemont Hospital, Montreal, Canada; and <sup>4</sup>Laboratory Medicine and Pathobiology, Faculty of Medicine, Toronto, Canada

### Summary:

**Our aim was to examine the relationship between mouthrinse matrix metalloproteinases (MMPs) and whole albumin levels (AL) relative to oral mucositis (OM) in allogeneic stem cell transplant (alloSCT) patients. Mouthrinse vertebrate collagenase levels are positively correlated with connective tissue destruction (CTD) in periodontitis and may also be involved in CTD associated with OM. Increases in salivary AL have been noted prior to OM onset and may serve as a predictive tool for OM and as a positive control in this study. A total of 23 alloSCT patients were visited eight times over 4 weeks following the transplant. OM was scored via a previously validated examiner-based ordinal system. Mouthrinse samples were collected and analyzed for MMP-1, 8, 13 (members of the vertebrate collagenase group) and AL. No significant correlation was found for MMP levels relative to OM scores. AL were positively and significantly associated with OM scores ( $P < 0.001$ ). MMP levels may not be an important factor in OM development and severity; however, mouthrinse AL may serve as a more objective measure of OM development and severity. *Bone Marrow Transplantation* (2005) 36, 33–38.**

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Oral mucositis (OM) occurs in 40–75% of patients undergoing allogeneic stem cell transplantation (alloSCT).<sup>1</sup> It is considered the most serious oral complication resulting from anticancer therapies<sup>2</sup> and can significantly affect morbidity, patient's tolerance of treatment and hence indirectly, survival and quality of life.<sup>3</sup> OM is thought to

be a consequence of several mechanisms. The most important may be the direct toxic effect of chemotherapeutic drugs and radiation therapy on the oral mucosa.<sup>4</sup> The clinical presentation of OM is variable but can be as severe as mucosal erosion progressing to ulceration and severe pain.<sup>5</sup> Generally, mucositis starts 5–7 days after the onset of chemotherapy.<sup>6</sup> The lesions reach maximum severity at days 11–14 after the onset of chemotherapy,<sup>6,7</sup> after which 90% resolution may be observed at 2–3 weeks post transplantation,<sup>8</sup> coinciding with cessation of the cytotoxic agents and return of the blood cell counts to normal ranges.<sup>9</sup>

The consequences and complications of OM are numerous and have an impact on outcomes of care, both clinical and economic. Patients with mucositis and neutropenia have a relative risk of bacteremia that is four times greater than individuals without mucositis.<sup>10</sup> These systemic infections may be life-threatening because of impaired immunologic defense mechanisms during the early post transplant period.<sup>11</sup> Approximately 25–45% of cases of septicemia in neutropenic cancer patients appear to originate from oral infection.<sup>12</sup> The pain associated with OM contributes to psychosocial distress, an inhibition of nutritional intake, impaired hydration, restriction of communication with others and premature withdrawal from therapy.<sup>13</sup> All of these can have a negative impact on survival. The magnitude of the problem of OM is compounded by the fact that there is no clearly defined prophylaxis or treatment, and supportive care for OM is mostly empirical.<sup>6</sup> This issue has been addressed quite extensively in the literature with the focus of managing OM being mainly symptom relief.<sup>6–8,12,14–16</sup> The etiology of OM is multifactorial; hence, approaches to prevention and management are also multifaceted.

### *Pathophysiology of OM and the relationship to matrix metalloproteinases (MMPs)*

Inherent in the current view of mucositis pathobiology is the concept of epithelial and extracellular collagen degradation as a central feature of inflammatory connective tissue destruction leading to OM.<sup>4,8,14,15,17</sup> In this regard, the MMP family of enzymes is involved in metabolic degradation of the extracellular matrix. It has been demonstrated that MMP-1, 8 and 13 (members of the

Correspondence: Dr I Shoval, Department of Periodontology, Faculty of Dentistry, University of Toronto, 124 Edward Street, Room 349, Toronto, Canada M5G 1G6; E-mail: irity2@yahoo.com  
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collagenase group of enzymes) are all involved in extracellular matrix degradation and remodeling during the course of periodontal disease (PD), and are positively associated with the magnitude of connective tissue and attachment loss.<sup>18–24</sup> Hence, it might be postulated that MMPs could play an important role in the development of OM.

#### *Mouthrinse albumin levels (AL) as possible predictor for OM occurrence and severity*

Serum albumin is one of the most predominant serum proteins in the oral cavity. A previous study found a 6–33-fold increase in whole salivary albumin concentration following alloSCT, which preceded the development of clinically detectable ulcers and correlated with its severity.<sup>25</sup> Thus, it was concluded that whole salivary AL may serve as a useful measure and predictor of this condition and hence an objective measure of lesion development. To our knowledge, the results of this study have not been duplicated elsewhere. Moreover, presuming that the above-noted data were reliable, measurement of oral AL could serve as a positive control for the mouthrinse assay study for MMP.

## Materials and methods

### *Patient population and recruitment*

Ethics approval for this project was obtained by University Health Network Ethics Review Board, Toronto, Ontario, Canada. Informed written consent was obtained from every patient participating in this study. These study participants were diagnosed with a hematological malignancy or condition that required treatment by alloSCT. A total of 23 patients were recruited from the Princess Margaret Hospital (PMH). All patients were over 18 years of age, and were competent in written and spoken English.

### *Baseline (BL) mouthrinse sampling and dental examination*

Two BL mouthrinse samples were obtained at the recruitment stage. Patients were asked to rinse with 3 ml of sterile saline for 10 s and expectorate. This was repeated after 120 s. Mouthrinses were centrifuged for 900 s at 2100 RPM at 4°C. The pellet was discarded and the supernatant was prepared to a 0.6 M salt concentration, aliquoted and stored at –70°C according to the protocol outlined previously.<sup>24</sup> All patients had a complete extra and intraoral dental examination that consisted of a full mouth series of radiographs, periodontal probing and scoring using the Canadian Periodontal Index of Treatment Needs (CPITN), scoring of decayed/missing/filled surfaces of teeth (DMFS) and a stimulated saliva flow measurement.

### *Patient visits and procedures*

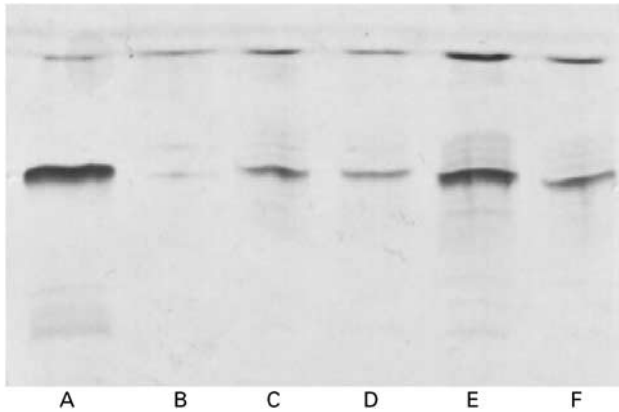
Patient visits were held on days 3, 7, 10, 14, 17, 21, 24 and 28 following the day of the alloSCT. Medical information gathered from the patients chart at each visit included current medications, presence of oral or systemic infection and laboratory results of daily blood work. Peripheral

blood cell counts, albumin, hemoglobin and platelets were recorded for correlation with biochemical measures of OM. The extent and severity of the OM was scored by one same examiner using a previously validated Likert scale (a discontinuous numerical scale, which permits grading of disease severity) called the Oral Mucositis Assessment Scale (OMAS), which was constructed by the Mucositis Study Group.<sup>26</sup> The OMAS was subsequently validated and used in a multicenter study.<sup>27</sup> It consists of a discontinuous scale that rates the extent of ulceration/pseudomembrane and erythematous areas in the mouth separately.

### *Albumin identification and quantification in oral mouthrinse samples*

The mouthrinse samples were allowed to thaw at room temperature (RT). Preparation of the sample for gel electrophoresis involved the addition of 8 µl TRIS buffer, 15 µl of 10% SDS and 10 µl of DTT to a 50 µl mouthrinse sample. In addition, 50 µl of 0.5 mg/ml bovine serum albumin (BSA; i.e. positive control) was combined with the same reagents as the mouthrinse sample. The mouthrinse samples and the BSA preparation were boiled for 300 s and then centrifuged for 40 s at 14 g. These were then analyzed using SDS-PAGE chromatography (PhastSystem, Pharmacia LKB Biotechnology, Uppsala, Sweden). Six sample lanes were run per gel, of which five lanes consisted of mouthrinse samples and the sixth lane had the BSA. In total, 6 µl of each mouthrinse sample or BSA preparation was deposited in each well (each sample run twice and an average taken) and the homogenous 7.5% Phast gel was run for 2700 s at 65 AvH. The protein bands were compared to the BSA standard such that comigration of Coomassie blue-stained bands in patient samples was considered as being indicative of the presence of albumin (Figure 1). Albumin was quantified with a computer densitometry program (FluorChem 8800, Instant Imager Electronic Autoradiography, Packard Meridian, CT, USA), using the BSA band at 0.5 mg/ml as a reference. The values for albumin quantity in the first and second mouthrinse were averaged.

To insure that albumin was being studied, a Western blot was performed as follows: mouthrinse samples obtained from three different patients as well as 0.5 mg/ml human serum albumin (HSA) was prepared for gel electrophoresis as detailed above. HSA and BSA differ by only 28 Da and comigrate on a 7.5% homogeneous Phast Gel. Following the same protocol for SDS-PAGE chromatography as above, the separated proteins were then thermally transferred to a nitrocellulose membrane using the Phast system (25 mA/gel for 1 h). Membranes were incubated with a 1:10 000 dilution of primary mouse monoclonal anti-human HSA IgG antibody (purchased from Sigma-Aldrich Inc., Saint Louis, MO, USA) for 1 h at RT. Following the washing step, a second incubation for 1 h was carried out with a 1:8000 dilution of secondary anti-mouse IgG antibody (Purchased from Sigma-Aldrich Inc., Saint Louis, MO, USA). This secondary antibody was labeled with horseradish peroxidase and could be detected with ECL Western blotting reagents. Omission of a primary antibody was used as a negative control.



**Figure 1** Coomassie blue stain following SDS-PAGE. Lane A, 66 kDa BSA 0.5 mg/ml. Lanes b-f, patient mouthrinse samples. Note the comigration of the bands (arrows) suggesting identity or similarity to BSA.

### MMP assay

A commercial assay kit (Chemicon International Inc. Temecula, CA USA) was utilized, which collectively detects MMP-1, 8 and 13. Multiple samples were analyzed simultaneously in a 96-well plate using spectrophotometric analysis (ICN Titertek Multiscan MCC/340, InterSciences Inc., Markham, Ontario, Canada). Each sample was analyzed twice and an average measurement was taken. This kit has a high level of sensitivity with respect to MMP detection (less than 5 ng of MMP/ml); however, it cannot distinguish between mammalian and bacterial collagenases, or between the MMP subtypes. This assay is based on a streptavidin-enzyme complex that is detected at an optical density (OD) read at 450 nm. MMP levels were calculated as a percentage relative to BL to account for interpatient variability as well as interkit (MMP assay kits) variability. In addition, mouthrinse MMP levels were normalized against mouthrinse AL by dividing absolute mouthrinse MMP levels by absolute mouthrinse AL. This was done to take into account the possibility of dilution of MMP levels due to leakage of serum proteins into the mouth.

### Statistical analysis

*A priori* sample size calculation for the number of study participants was based on the study by Mancini *et al.*<sup>24</sup> who studied MMP levels in patients with periodontitis. Using periodontitis as a model, we assumed that mouthrinse MMP levels would rise similarly in alloSCT patients suffering from OM. This resulted in a requirement of a sample size of 10 patients (alpha level=0.05 and Power=90%, two-tailed correlations). This was doubled to 20 to account for patients who may not have wanted to, or not have been able to continue the study due to sickness or possible death. In total, 23 patients were included in the study.

Pearson's correlation coefficient was used to examine the relationship between mouthrinse MMP levels and OMAS scores relative to BL, as well as mouthrinse MMP levels and peripheral neutrophil counts. Owing to the fact that the distribution of mouthrinse AL was positively skewed,

Spearman's correlation coefficients were used to examine the relationship between mouthrinse AL and OMAS scores, as well as mouthrinse AL and serum AL.

## Results

### Population characteristics

The mean age of the 23 patients included in the study was  $42.2 \pm 11.9$  years. Seven patients (30%) were diagnosed with AML, five patients (22%) with CML, four patients (17%) with ALL, one patient (4%) with CLL and six patients (26%) suffered from other hematological conditions. In all, 21 patients had total body irradiation included in the preparative regimen. The oral mucosal lesions in this patient group reached maximum severity between days 9 and 18 post-alloSCT, after which the lesions began to improve and disappear.

### Dental examination

The average number of teeth present in this group of patients was 25.6 with a standard deviation of 7.7. One patient was completely edentulous and one patient was completely edentulous in the maxillary arch. Six patients had a Hickman line at the BL examination, which prevented periodontal probing. Of the 17 remaining patients, five of these had evidence of mild to moderate periodontal disease based on radiographs, the CPITN score and visual examination. It is important to note at this point that MMP levels for each patient were measured relative to BL and this data was used to analyze the MMP data. This was done in order to eliminate the variability that may result as a consequence of periodontal disease.

### Mouthrinse MMP levels and the relationship to OM

MMP levels were calculated as a percentage relative to BL to account for interpatient variability as well as interkit (MMP assay kits) variability. There was no observable correlation with respect to the development of oral ulcerations, between MMP levels and OM. Pearson's correlation coefficient for mouthrinse MMP levels relative to BL vs mean OMAS scores relative to BL was 0.12 with a *P*-value of 0.143 ( $n = 148$  data points).

### Mouthrinse AL and the relationship to OM

A significant trend towards increasing AL over time was noted in the post transplant period. Mouthrinse AL paralleled the onset and severity of the oral lesions but did not predict their onset (Table 1 and Figure 2).

Spearman's correlation coefficient for mean mouthrinse AL vs mean OMAS scores was significant regardless of whether the data were analyzed cross-sectionally or relative to BL. This coefficient was 0.49, *P*-value < 0.001 ( $n = 179$  data points) for data analyzed cross-sectionally, and  $r_s = 0.50$ , *P*-value < 0.001 ( $n = 155$  data points) for the data analyzed relative to BL.

### Mouthrinse MMP levels/mouthrinse AL and the relationship to OM

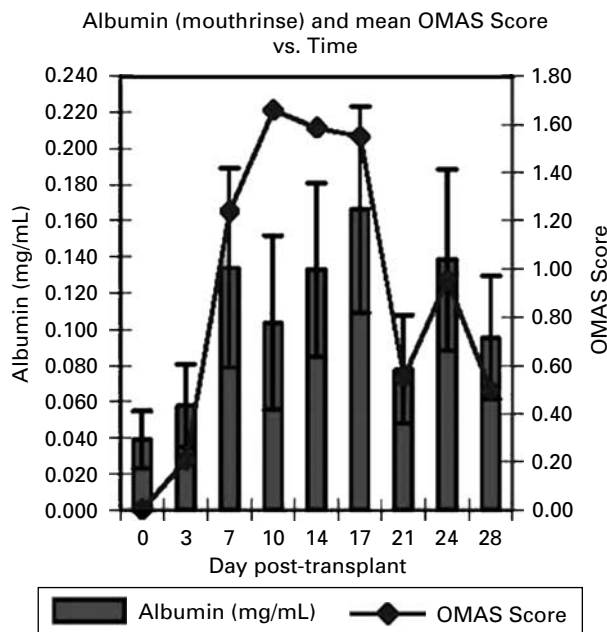
Mouthrinse MMP levels were normalized against mouthrinse AL by dividing absolute mouthrinse MMP levels by absolute mouthrinse AL, so as to take into account the possibility of dilution of MMP levels due to leakage of serum proteins into the mouth. Using the Spearman's correlation coefficient, there was a significant correlation between MMP/AL and mean OMAS score. ( $r = -0.48$ ,  $P$ -value  $< 0.001$ ,  $n = 171$  data points).

### The relationship between mouthrinse and serum proteins

A statistically significant negative Spearman's correlation coefficient of  $-0.351$  ( $P$ -value  $< 0.001$ ,  $n = 179$  data points)

**Table 1** Mean OMAS Score ( $\pm$  standard error of mean) and mean mouthrinse albumin score ( $\pm$  standard error of mean) relative to the day post transplant

Day post transplant	Mean OMAS score ( $\pm$ standard error of mean)	Mean mouthrinse albumin (mg/ml) ( $\pm$ standard error of mean)
0	0	$0.039 \pm 0.016$
3	$0.21 \pm 0.10$	$0.058 \pm 0.023$
7	$1.24 \pm 0.26$	$0.134 \pm 0.055$
10	$1.66 \pm 0.34$	$0.104 \pm 0.048$
14	$1.58 \pm 0.38$	$0.133 \pm 0.048$
17	$1.55 \pm 0.42$	$0.166 \pm 0.057$
21	$0.55 \pm 0.26$	$0.078 \pm 0.030$
24	$0.95 \pm 0.34$	$0.138 \pm 0.050$
28	$0.50 \pm 0.23$	$0.095 \pm 0.034$



**Figure 2** Mean ( $\pm$  standard error) mouthrinse AL and mean OMAS scores with time after transplant ( $n = 23$ ). Both scores parallel one another, suggesting that OM lesion severity parallels oral albumin leakage into the oral cavity.

was derived for mouthrinse AL vs serum albumin. Mouthrinse MMP levels relative to BL were positively and significantly (Pearson's correlation coefficient of 0.17,  $P$ -value = 0.034,  $n = 148$  data points) correlated with serum neutrophil counts relative to BL.

## Discussion

### Collagen degradation/MMP activity relative to OM

Collagen is the major structural connective tissue protein in the body and also comprises a major component of the tissues that support and invest the teeth. MMP levels have been shown to parallel connective tissue destruction in the periodontium.<sup>19–21,23,24,28–31</sup> Hence, one of the objectives of this research was to investigate if MMPs may play as important a role in the pathophysiology of oral mucosal lesions observed post-alloSCT as they do in periodontal disease. The results of the study suggest that MMPs do not necessarily play a role in the development of OM as in other diseases characterized by destruction of connective tissues such as periodontitis. To our knowledge, the relationship between oral MMP levels and OM in alloSCT patients has not been investigated. However, Collin *et al*<sup>32</sup> studied salivary MMP-8 and MMP-9 (levels and activity) in patients with type 2 diabetes mellitus, and incidentally noted occurrence of oral ulcers and stomatitis. This occurrence did not correlate to MMP level or activity, similar to our findings suggested here.

There are several reasons that may account for our findings, and further study of this issue is still required. The assay used in this study measured MMPs-1, 8 and 13 levels concurrently. Given the putative differential roles for these MMPs, it is possible that the pathophysiology of OM could still be related to changes in specific host MMP levels, or perhaps totally different MMPs than those measured here. Moreover, this assay is unable to distinguish between mammalian and bacterial collagenase. Given the almost certain contribution of gram negative organisms in OM, there are almost certainly going to be measurable levels of bacterial collagenase which could mask changes in mammalian (host) collagenase levels. When peripheral neutrophil counts were correlated to MMP levels (both parameters analyzed relative to BL levels), a statistically significant correlation coefficient was noted. This could suggest that bacterial collagenases did not play as large a role as they might have in MMP measurements. However, the coefficient of 0.174 is not very strong and must be interpreted accordingly in that the assay we used measures three MMPs and not only neutrophil-associated MMP-8, as noted above.

### AL and OM

Over the post transplant period, mouthrinse AL increased significantly and correlated moderately well with both serum AL (negative correlation) and OM severity (positive correlation). This phenomenon is likely reflective of 'leakage' of serum proteins into the mouth from the ulcers. Contrary to the findings by others,<sup>25</sup> the data shown here

did not demonstrate increases in mouthrinse AL prior to clinical detection of the ulcers. This may be related to a number of factors including the way the mouthrinse samples were collected and/or analyzed. In previous studies, stimulated saliva was collected<sup>25</sup> and analyzed using low-level radial immunodiffusion. In this investigation, protein gels were stained with Coomassie blue and quantified by densitometry. Perhaps silver staining would have been more sensitive (than Coomassie blue), thereby allowing the identification of earlier increases in oral albumin. Moreover, it is likely that mouthrinse samples contain less protein than stimulated whole saliva collection. However, utilization of a mouthrinse is almost certainly less invasive and bothersome and more humane for a patient as compared to obtaining whole saliva, and that is why this method of collection in this patient group was chosen. Regardless, the data show that mouthrinse AL closely parallel oral mucosal lesions and can serve as a rapid, reliable and noninvasive measure of OM onset and severity without having to resort to subjective or more invasive measures.

It is important to note the correlation between increased OMAS scores and increased mouthrinse albumin scores. This finding can act as a surrogate for or supplement the clinical assessment of oral mucositis. An OMAS score of >1 was always associated with an albumin concentration >0.1 mg/ml. Hence, it can be assumed that albumin concentrations of >0.1 mg/ml are associated with more severe mucositis.

Serum AL decreased from BL to day 3 following which levels remained constant. A decrease in serum albumin has been noted in critically ill patients and is related to an increase in capillary leakage.<sup>33</sup> When serum AL were correlated to mouthrinse AL, a significant negative correlation was noted ( $r = -0.351$ ,  $P$ -value < 0.001), and this could suggest that oral albumin leakage is a significant source of serum albumin loss.

When MMP levels were normalized against albumin, there actually appeared to be a significant and *negative* relationship with OM. This would suggest that MMP levels likely play less of a role than predicted since when we normalize for the amount of 'leakage' of proteins into the oral cavity, there appears to be even less MMP.

In conclusion, analysis of relative levels of MMP-1, 8 and 13 together suggests that they do not appear to play a functional role in OM development and progression in alloSCT patients. However, when MMP levels were normalized against albumin, there actually appeared to be a significant and *negative* relationship with OM. Mouthrinse AL parallel OM onset, development and resolution quite closely and can therefore serve as a tool to measure OM rather than resorting to more subjective or invasive approaches to measurement.

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