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The effect of parotid gland-sparing IMRT on salivary composition, flow rate and xerostomia measures

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Abstract

Objectives—To describe parotid gland (PG) saliva organic and inorganic composition and flow rate changes, after curative intensity-modulated radiotherapy (IMRT) for head and neck cancer (HNC) and analyse the relationship between PG saliva analytes and xerostomia measures.

Methods and Materials—Twenty-six patients recruited to five prospective phase 2 or 3 trials which assessed toxicity and efficacy of IMRT by HNC subsite, provided longitudinal PG salivas. Salivary flow rate, subjective and objective xerostomia measures were prospectively collected and salivas tested for inorganic and organic analytes. Statistical comparisons of longitudinal analyte changes and analysis for a relationship between dichotomised xerostomia score and saliva analytes were performed.

Results—One-hundred and forty-two PG saliva samples from twenty-six patients were analysed. At 3–6 months after IMRT, stimulated and unstimulated salivas showed significantly decreased flow rate, total protein (TP) secretion rate, phosphate concentration and increased lactoferrin (LF) concentration. Stimulated salivas alone had elevated LF secretion rate and beta-2-microglobulin (B₂M) concentration with decreased Calcium (Ca²⁺) and Magnesium (Mg²⁺) concentrations and Ca²⁺ secretion rate. At >12 months, under stimulated and unstimulated conditions, increased LF concentration and decreased Mg²⁺ and phosphate concentration persisted and, in stimulated saliva, there was decreased potassium (K⁺) and Mg²⁺ concentration. Unstimulated TP secretion rate was

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Conflicts of interest statement

Thomas M Richards – No conflicts to declare.

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lower in the presence of high grade xerostomia. Otherwise, no relationship between xerostomia grade and PG salivary flow rate, TP and Ca^{2+} secretion rate was found.

Conclusion—Fewer significant differences in PG saliva analytes >12 months after IMRT indicate good functional recovery. Residual xerostomia after IMRT will only be further reduced by addressing the sparing of subsites of the PG or other salivary gland tissues, in addition to the PG.

Keywords

Radiotherapy; head and neck cancer; saliva; parotid gland; xerostomia; IMRT; toxicity

Introduction

IMRT is commonly delivered for the primary or combined modality curative therapy for squamous cell cancer of the head and neck (SCCHN). The PG are frequently in close proximity to the tumour (Standring, 2005), and this leads to incidental irradiation manifesting as acute and late toxicity. Salivary gland hypo- function has been described as a combination of three unique but inter-related factors (Nederfors, 2000), xerostomia, defined as the subjective feeling of dry mouth, reduced saliva flow and altered saliva composition. Most studies investigating PG late toxicity after RT have been based on salivary flow or xerostomia score. The mechanism by which radiotherapy (RT)-induced salivary gland (SG) toxicity causes xerostomia is currently thought to be through reduced salivary flow (Vissink et al., 2010). The changes in PG salivary composition after IMRT and the relationship of individual components with xerostomia is unknown.

Several unanswered questions remain, including the nature of long-term changes in parotid saliva composition after IMRT and potential associations between individual salivary components and xerostomia measures. We present an analysis of the PG salivary inorganic and organic saliva composition from samples collected during 5 prospective clinical trials of IMRT. The associated PG radiation dosimetry, salivary flow and xerostomia data provides a detailed investigation of PG function after IMRT.

Material and Methods

Patients and treatment

Patients recruited to five prospective phase II and III clinical trials of PG-sparing IMRT for HNC were screened for eligibility to this study. The aim of these trials was to assess the effect of PG-sparing IMRT, by primary tumour sub-site, on quantitative and qualitative measures of SG function and to report survival outcomes, as described in more detail previously (Nutting et al., 2011, Miah et al., 2012, Miah et al., 2015, Zaidi et al., 2011). Patients and prospectively collected saliva samples were identified from the Royal Marsden Hospital (RMH) head and neck RT clinical trials log and sialometry database. Patients were eligible if they met the following criteria: previously treated with curative, high dose IMRT $\pm$ chemotherapy (CT) for HNC: had a baseline saliva sample collected using a standardised protocol from one or both PGs before CT- IMRT and induction CT (if administered); had completed 6 months of follow-up since the end of IMRT; had a saliva sample from the same PG at 1 sequential time-point, 3- to 24-months after IMRT; had an adequate sample

volume, >150 µL for full composition analysis; and had documented PG salivary flow rate to allow secretion-rate calculation. The current study was designed after commencement of all 5 prospective studies. All saliva samples collected prospectively within each of our centres IMRT trials used a standardised collection protocol. To assess our study hypothesis and provide data in a cost-effective and timely manner we proposed a combined retrospective analysis of data and samples from the 5 prospective studies already recruiting patients at our centre.

The IMRT planning and delivery technique for each trial has been previously described (Nutting et al., 2011, Miah et al., 2012, Miah et al., 2015, Urbano et al., Miah et al.). The 5 clinical trials were approved by the institutional research and ethics committee RMH CCR and registered on [ClinicalTrials.gov](https://clinicaltrials.gov) (clinical trials registration number) - CCR1978 (NCT02055989), CCR2059 (NCT00081029), CCR2588 (NCT02149602), CCR2608 (NCT02149641) and CCR3301 (NCT02068313). All studies except CCR3301 have completed accrual and are either in follow-up or closed. All patients signed a written informed consent, including optional consent for storage and analysis of saliva samples. The study was performed in accordance with the Declaration of Helsinki.

Saliva collection and toxicity assessment

The recommended method of sialometry has been previously described (D'Hondt et al., 1998, Ship et al., 1997). Stimulated (2% citric acid, 2004-11 (RMH pharmacy) or 1.8% citric acid pastilles, 2011-12 (Simpkin's, Sheffield, UK)) and unstimulated PG salivas were collected on ice, from bilateral PGs, using Lashley cups. Salivas were collected between 10 am and 3 pm, patients were advised not to eat, drink or smoke for 1 hour prior to saliva collection. Flow rates were determined gravimetrically (1 g = 1 mL). Saliva was stored at -80°C until analysis.

Late toxicity scores (Radiation Therapy Oncology Group (RTOG)/European Organization for Research and Treatment of Cancer and Late Effects in Normal Tissues-Subjective, Objective, Management, and Analytic scale (LENT-SOMA)) assessed normal tissue toxicity later than 3 months after completion of RT. This xerostomia measure is a reliable and validated scale for scoring late toxicity in head and neck cancer (Ho et al., 2010). Subjective xerostomia of grade 2 or worse was "partial but persistent oral dryness or worse". This was assessed by clinician directed questioning of the patient and visual oral examination recorded at 3, 6, 12, 18, and 24 months follow-up (Rubin et al., 1995).

Inorganic and organic composition analysis

Total protein (TP) concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard. This is described in detail elsewhere (Smith et al., 1985).

Individual proteins, secretory IgA (sIgA), beta-2-microglobulin (β_2 M) and lactoferrin (LF) were analysed using a sandwich enzyme-linked immunosorbent assay (ELISA) technique as previously described (Carpenter et al., 2000) with some modifications. Ninety-six well microtitre plates (Iwaki, Tokyo, Japan) were coated with polyclonal rabbit anti-sIgA antibody (1:1000) (A0262, Dako Labs Ltd, High Wycombe, UK), polyclonal rabbit anti-

human $\beta_2\text{M}$ (1:2000) (A0072, Dako Labs Ltd, High Wycombe, UK) or polyclonal rabbit anti-human LF (1:1000) (ab15811, Abcam, Cambridge, UK). Equal saliva sample volumes (duplicate) were added at a concentration of 1:50. Standards (duplicate) of purified proteins (0.1 $\mu\text{g/mL}$ $\beta_2\text{M}$ (from urine, Sigma, UK), 1 $\mu\text{g/mL}$ sIgA (from breast milk, Sigma, UK) and 0.8 $\mu\text{g/mL}$ LF (from breast milk, Sigma, UK)) were used. Samples and standards were serially diluted down the plate and incubated for 1 hour at 37°C. Secondary antibodies (all rabbit anti-human linked to horseradish peroxidase (HRP)) were $\beta_2\text{M}$ (1:3000), (P0174, Dako Labs Ltd, UK), sIgA (1:10,000) (P0216, Dako Labs Ltd, UK) or LF (1:10,000) (pp062, The Binding Site, UK) linked to horseradish peroxidase (HRP). A detection solution of 3,3',5,5'-tetramethylbenzidine (TMB), pH 5.5, was added to each well. Reaction times for $\beta_2\text{M}$, sIgA and LF of 8.5, 6 and 10 minutes were determined from earlier experiments (data not shown). The reaction was stopped by adding 2 M H_2SO_4 and colour intensity was measured photometrically at 450 nm in a micro-plate reader (Model 3550, BioRad Lab., UK).

Selected inorganic components, calcium (Ca^{2+}), sodium (Na^+), magnesium (Mg^{2+}), potassium (K^+), and Phosphate (PO_4^{3-}), were analysed by inductively-coupled plasma-mass spectrometry (ICP-MS; Elan series, Perkins-Elmer, MA, USA). This technique has previously been described (Houk & Thompson, 1988). Briefly, patient samples were diluted 1:100 in double-distilled H_2O to a final volume of 1 mL, frozen at -80°C and transferred by courier on dry ice to the Kings College London mass-spectrometry facility for analysis. Samples were defrosted at room temperature and immediately analysed by ICP-MS according to the operating protocol defined by the manufacturer.

Statistical Analysis

Parametric data were presented as mean ($\pm 95\%$ confidence interval) and non-parametric data as median ($\pm$ inter-quartile range) values. The D'Agostino and Pearson test was used to assess normality of data distribution.

Intra-patient changes at baseline, between unstimulated and stimulated PG saliva flow rate, concentration and secretion rate, were analysed with paired t-tests (Gaussian data) or Wilcoxon's test (non-Gaussian data), p-value <0.05 statistically significant.

Analysis of changes between baseline and early (3-6 months) or late (>12 months) time-points were performed using paired t-tests (Gaussian data) or Wilcoxon's test (non-Gaussian data). A p-value of <0.025 (Bonferroni correction applied) was deemed statistically significant.

Exploratory analysis of the salivary analytes (TP, Ca^{2+} , PG salivary flow rate), implicated in the aetiology of xerostomia, was performed. Two physician-reported xerostomia measures (LENT-SOMA and RTOG scales) were assessed for a possible relationship with each analyte. Xerostomia score was dichotomised, high grade (HG, grade 2) or low grade (LG, grade 0-1). Change in analyte measure at early (3-6 months) time-point was performed in relation to the dichotomised xerostomia score. A p-value <0.05 was deemed significant. The rate of high grade xerostomia at the late (12-24 months) time-point was low, so no valid analysis was possible. Sample size was not calculated in advance however we used all

eligible samples over the 8 year period of prospective trial recruitment to maximize statistical power.

Statistical analysis was performed using GraphPad Prism v6.0, GraphPad, San Diego, Ca, USA (www.graphpad.com).

Results

Twenty-six patients from 5 prospective clinical trials provided sequential PG salivas for analysis. Baseline patient and treatment demographics are shown in table 1. One-hundred and forty-two PG salivas were analysed with slightly more stimulated salivas at each time-point. The stimulation collection technique and number of samples by time-point are reported in table 2. At baseline two patients provided bilateral unstimulated PG salivas, total of 28 samples and seven patients provided bilateral stimulated PG salivas, total of 33 samples. Unstimulated then stimulated saliva collection was planned at each time point however not all patients provided both samples, therefore the mean number of samples per patient was 5.5. The primary tumour site was larynx, oropharynx or hypopharynx in 20 (76.9%) patients. Patient compliance was good with >90% of patients receiving the prescribed treatment in the planned timeframe (<7 day extension) and <5% of patients were lost to follow-up.

Baseline salivary composition and flow rate

The effect of salivary stimulation on the concentration of PG saliva analytes was assessed at baseline (table 3). Patients with paired unstimulated and stimulated specimens were included in this analysis.

Baseline salivary analyte concentration and flow rate showed two significant changes following salivary stimulation, a 2.5-fold increase in mean PG saliva flow rate ($p<0.001$) and an increase in potassium concentration ($p=0.001$). Salivary secretion rates for all proteins and Ca^{2+} were significantly increased in stimulated vs unstimulated salivas. The fold increases in mean secretion rates with stimulation for TP, sIgA, B2M, LF and Ca^{2+} were 2.4 ($p=0.004$), 2.3 ($p=0.002$), 2.2 ($p=0.002$), 3.1 ($p=0.01$) and 2.2 ($p=0.002$), respectively.

Organic analyte salivary composition after IMRT

Saliva samples, collected under stimulated and unstimulated conditions under both stimulation conditions, were analysed for organic composition (table 4a).

Unstimulated PG saliva TP concentrations and secretion rates were significantly reduced in 3-6 month samples. Both returned to normal at >12 months. Secretory IgA showed no significant change in concentration at early and late time-points, and a numerical reduction in secretion rate at 3-6 months ($p=0.09$) which recovered at >12 months. In early samples, B2M showed a trend to increase in concentration ($p=0.04$) with recovery at 12-24 months. Finally, LF concentration was significantly increased for early and late samples ($p=0.003$ and $p=0.02$, respectively). However, LF secretion rate was unaltered from baseline.

Stimulated PG saliva TP concentration was not significantly changed, at either time point. Secretion rates were, however, significantly reduced in early samples ($p=0.002$) but normalised at >12 months. Secretory IgA showed no significant change in concentration at early or late time-points, but a trend to reduction in secretion rate for 3-6 month samples ($p=0.039$), recovering at >12 months. The B₂M concentration in early samples increased ($p=0.001$), with recovery to baseline at >12 months. However, the B₂M secretion rate at both time-points was not significantly altered. Finally, LF concentration was significantly increased, in early and late samples, $p<0.001$ and $p=0.02$, respectively. The secretion rate was also significantly increased at 3-6 months ($p=0.009$) with a maintained trend to increase at >12 months, ($p=0.037$).

Inorganic analyte salivary composition after IMRT

Saliva samples, collected under both stimulation conditions, were analysed for inorganic composition (table 4b).

Unstimulated PG saliva Ca²⁺ concentrations and secretion rates were unchanged at early and late time-points. The Ca²⁺ secretion rate at 3-6 months showed a trend to reduction ($p=0.04$), returning to baseline values at >12 months. Neither the PG saliva Na⁺ nor Mg²⁺ concentrations were significantly changed, at either time-point. However, at >12 months, the Mg²⁺ concentration showed a trend to reduction ($p=0.03$). The K⁺ concentration was significantly increased ($p=0.008$) at 3-6 months and returned to baseline at >12 months. Finally, phosphate concentration was significantly decreased at both time-points, $p<0.001$ and $p=0.008$, respectively.

Stimulated PG saliva Ca²⁺ concentration and secretion rate were significantly lower at 3-6 months, both $p<0.001$. At >12 months both had returned to baseline. Na⁺ concentration showed a trend to increase in early samples ($p=0.047$), but unchanged from baseline at >12 months. Mg²⁺ concentration was significantly reduced at both time-points, $p=0.003$ and $p<0.001$, respectively. K⁺ concentration was not significantly changed at 3-6 months but significantly decreased in late samples ($p=0.004$). Finally, phosphate concentration was significantly decreased for early and late samples, both $p<0.001$.

Relationship between xerostomia and PG salivary flow and composition

The frequency of LENT-SOMA scale HG xerostomia at each time point (BL, 3m, 6m, 12m, 18m, and 24m) was 0%, 53.8%, 30.8%, 22.7%, 11.1% and 6.3% respectively.

The frequency of RTOG scale HG xerostomia at each time (BL, 3m, 6m, 12m, 18m, and 24m) was 0%, 34.6%, 24%, 17.4%, 11.1% and 12.5% respectively.

An exploratory analysis for PG salivas at 3-6 months after IMRT was performed to assess for a relationship between PG salivary flow rate, TP and Ca²⁺ secretion rate vs. dichotomised, physician-reported xerostomia rate (HG vs LG).

Unstimulated PG salivary flow rate (ml/min; baseline = 0.14) was lower for patients where HG xerostomia was present. For LENT-SOMA scale at 3-6 months, flow rates were 0.05 (HG) vs. 0.1 (LG), $p=0.24$ and for the RTOG scale flow rates were 0.04 (HG) vs. 0.1 (LG),

$p=0.25$. Unstimulated TP secretion rate (mg/min; baseline = 0.16) were reduced for patients where HG xerostomia was present. For the LENT-SOMA scale secretion rates were 0.05 (HG) vs. 0.09 (LG), $p=0.29$ and for the RTOG scale 0.03 (HG) vs. 0.09 (LG), $p=0.017$. Finally, unstimulated Ca^{2+} secretion rate (nmol/min; baseline = 165.8) was lower where HG xerostomia was present. For the LENT-SOMA scale secretion rates were 51.2 (HG) vs. 136.1 (LG), $p=0.096$ and for the RTOG scale 27.2 (HG) vs. 52.4 (LG), $p=0.18$. No statistically significant relationship was seen between stimulated PG salivary flow rate, TP secretion rate or Ca^{2+} secretion rate and the dichotomised xerostomia score.

Discussion

Changes in SG function induced by radiotherapy have been assessed in the present study. Use of a PG- sparing IMRT technique is associated with early reduction in unstimulated and stimulated PG saliva flow, alongside early and some persistent changes in PG saliva composition. The statistically significant, longitudinal changes in PG saliva parameters are summarised (tables 5a and b). Most were seen in early, stimulated salivas, specifically a decreased Ca^{2+} , Mg^{2+} , and phosphate concentration, decreased TP and Ca^{2+} secretion rate and increased LF and B_2M concentration and LF secretion rate. At >12 months, the increased LF concentration and the reduction in Mg^{2+} and phosphate concentration persisted. A decrease in K^+ concentration was also detected. Fewer significant differences at >12 months suggest that SG function recovered well in quantity and quality.

The changes seen may be of clinical relevance when the function of each analyte is considered. Acinar cell dysfunction was detected at 3-6 months, under both stimulated and unstimulated conditions, evidenced by the significant reduction in the secretion of acinar cell-derived components; salivary fluid and TP (unstimulated) and salivary fluid, TP and Ca^{2+} (stimulated). The elevation of LF (unstimulated and stimulated) and B_2M (stimulated) concentration may be partly accounted for by the reduced fluid volume with raised concentration of these solutes. The increased LF secretion rate, incorporating flow rate, indicates an up-regulation of this specific protein.

A further consideration is the anti-infective or immune response-related roles of organic solutes in saliva, specifically sIgA, LF and B_2M . Oral mucositis, inflammation, tumour necrosis and oro-pharyngeal infections are commonly seen sequelae of RT for HNC. Increased secretion of immune response molecules could occur in response to local cellular damage in the PG, or possibly related to oral mucosal damage. This may be a reason for the elevated LF secretion in early stimulated samples. Additionally, as no significant change in sIgA secretion rate was seen, this indicates a relative up-regulation of this protein, as the TP concentration and secretion rate was reduced, supporting this hypothesis. Evidence for ductal cell function is related to the salivary Na^+ concentration, this remained unchanged in this study despite a reduction in salivary flow, providing evidence of dysfunction. Phosphate was the only analyte persistently reduced at both time-points under both stimulation conditions. However, its mechanism of secretion and transport in saliva is unclear.

There are potential clinical implications of the reduced TP and Ca^{2+} secretion rates and phosphate concentration, since these may increase the susceptibility of patients to dental

caries. Jensdottir *et al* (Jensdottir et al., 2013) reported that when acidic candies, with or without added Ca^{2+} , were administered to HNC patients after RT, the whole mouth saliva (WMS) saliva flow increased. For candies with added Ca^{2+} , the saliva had a significantly lower erosive potential in an *in vitro* model. Bardow *et al* (Bardow et al., 2005) showed *in vivo* that high unstimulated WMS concentrations of phosphate, TP and amylase were protective against caries. Finally, Garcia-Godoy *et al* (Garcia-Godoy & Hicks, 2008) reported that Ca^{2+} , phosphate and fluoride derived from saliva and sequestered in to the dental bio-film are required for effective remineralisation and maintenance of the enamel surface integrity. These studies support the hypothesis that a lower Ca^{2+} , TP and phosphate secretion rate is associated with dental caries susceptibility after RT for HNC. Our data suggests that for optimal dental caries prevention, despite recovery of parotid saliva flow, prolonged oral care with, for example, a mouth-wash supplemented with Ca^{2+} , phosphate and fluoride, may be necessary.

The data presented here represent the first assessment of changes in individual SG secretion and composition of salivas collected following IMRT, under unstimulated conditions. They provide the most comprehensive analyte coverage for saliva composition of any published study, whether ductal or whole mouth saliva and the largest prospectively collected cohort for the assessment of salivary composition changes >12-months after any RT technique.

Twelve historical studies investigated saliva composition (tables 6a and b), 9 describe changes in WMS (table 6b) and 11 studied patients treated with non-conformal RT technique (2D-RT). SG hypo-function is almost inevitable after 2D-RT for HNC, so these studies are of little relevance to modern radiotherapy practice.

The early reduction in PG saliva flow rates (table 4a) relative to baseline (100%); unstimulated (30.1%) and stimulated (44.4%) with recovery at >12 months, unstimulated (87%) and stimulated (65.3%) were similar to that seen in 2 recent prospective phase III trials (Kam et al., 2007, Pow et al., 2006). No studies investigating unstimulated PG saliva composition after RT are reported therefore, the data in this study provides novel findings.

Two studies (Funegard et al., 1994, Valdez et al., 1993), similar to ours, analysed stimulated, individual ductal PG salivas following conventional 2D-RT. Despite limitations of comparing contemporary data with studies from the early 1990s, where a non-conformal RT technique was used, some similarities were seen. Specifically, there was an early reduction in stimulated PG saliva flow and increased K^+ concentration. At >12 months, there was increased LF concentration. However, differences reported by Funegård *et al* (Funegard et al., 1994) were an early increased TP concentration, but no significant changes in any parameter >12 months. A third publication (Randall et al., 2013) was the only to assess IMRT-treated patients. It differed from our study by collecting WMS only. Prospective follow-up was limited to six weeks with no alteration in Ca^{2+} or mucin (Muc5b) concentration reported, just significantly reduced WMS flow rate.

Reviewing all published studies, it is not possible to draw definitive conclusions regarding the changes in salivary composition after RT, due to heterogeneity in analytes selected for testing and short follow-up in the more statistically robust prospective studies. The one area

of consensus for all studies reporting salivary flow(Funegard et al., 1994, Randall et al., 2013, Tiwana et al., 2011, Makkonen et al., 1986, Brown et al., 1976, de Barros Pontes et al., 2004, Ben-Aryeh et al., 1975, Almstahl & Wikstrom, 2003) was a significant reduction in either WMS or individual parotid saliva flow at <6-months. At >12 months, Valdez *et al*(Valdez et al., 1993) reported persistently reduced PG and SMG saliva flow rates, but others(Funegard et al., 1994) report recovery of PG flow.

Van Luijk et al show that if the main excretory parotid duct region is spared radiation dose then improved recovery of a glandular function was seen after radiotherapy (van Luijk et al., 2015). These data provide the first data in humans that sparing the stem cell region of the PG reduces the rate of xerostomia. In our study the PG radiation dose constraint was the whole PG mean dose. Specific subsites of the parotid gland were not given additional weighting during IMRT plan optimization but a gentle gradient in radiation dose was seen across the PG from higher in medial PG to lower laterally, in regions furthest from the target volume.

Investigation for a correlation between the tumour site and the impact on PG saliva parameters would be interesting but was not planned in our study. An assessment of this was unfortunately not feasible due to small numbers of patients per HN subsite. IMRT treatment of lateralised or bulky primary tumours, particularly in the oropharynx is likely to cause higher mean PG doses due to overlap of the gland with the RT target volume. This will cause a significant detrimental effect on saliva flow and ductal function. The aim however of this study was to assess patients where they could provide >150 µl of saliva therefore the PG mean radiation dose of 28.2 Gy (unstimulated) and 29.7 Gy (stimulated) was close to the recommended tolerance.

Xerostomia measures are reported in three studies (Dijkema et al., 2012, Randall et al., 2013, Tiwana et al., 2011). No studies have undertaken an analysis for a possible relationship between individual saliva components, and physician-(Tiwana et al., 2011) or patient-reported(Dijkema et al., 2012, Randall et al., 2013) xerostomia scores. We assessed for a possible relationship between PG saliva analytes and xerostomia severity after IMRT. At 3-6 months, patients with HG xerostomia (RTOG scale) showed a significantly lower recovery of unstimulated TP secretion rate. Furthermore, the flow rate and Ca^{2+} secretion rates (unstimulated) showed a numerical decrease in analyte recovery for patients with HG vs. LG xerostomia. A relationship between these three parameters and the frequency of HG xerostomia, under stimulated conditions, was not evident. The fall in Ca^{2+} secretion rate, in sequential samples, and the lower recovery of Ca^{2+} secretion rate in patients with HG xerostomia are interesting. Ca^{2+} has previously been shown to be involved in mucin-mucin cross-link formation(Raynal et al., 2003) at the buccal mucosal surface. Ca^{2+} , therefore, may also be crucial in the prevention of xerostomia, besides its protective role against dental caries. At 3-6 months after IMRT, a trend for a lower recovery in secretion rates of TP, Ca^{2+} and PG flow, in patients with HG xerostomia, was found for unstimulated but not stimulated samples. For most of the time, PGs are in the unstimulated state and, so, this finding may be clinically important.

Potential limitations of this study include the use of chemotherapy in 58% of patients. However, to date, there is no clear evidence that platinum-based CT has a detrimental effect

on SG function. A recent publication (Miah et al., 2013) reports no significant difference in acute, <3 months, and late, >3 months HG physician-reported xerostomia with the addition of CT to IMRT. A post-hoc analysis of our data showed no significant difference in PG saliva flow rate for unstimulated or stimulated samples with the use of concomitant chemotherapy. Another limitation is that, despite prospective sample collection, this study was retrospectively planned and, therefore, did not include patients who had no adequate saliva specimen available for numerous reasons. The frequency of no or very low PG saliva flow (<0.03 mL/min) was 35-40%. The eligibility rate was therefore under 10% of IMRT treated trial patients. The data presented must, therefore, be viewed as representative of patients with a residual PG flow rate of >0.03 mL/min and where a mean, whole PG radiation dose of <30 Gy was achieved. This is expected to be met for most HNC patients treated with PG-sparing IMRT therefore we consider that the data presented can be generalised to all patients with residual or recovered salivary flow >0.03 mL/min. The authors believe that the reasons for failure of data collection/samples e.g disease progression or death or patient declined procedure, would not bias the results as they would be distributed randomly within the >300 potentially eligible patients however, we cannot entirely exclude the chance of inclusion bias due to low numbers but feel that this risk is low.

Salivary flow and xerostomia scores have been utilised in several previous prospective studies of IMRT (Pow et al., 2006, Kam et al., 2007, Marucci et al., 2012) and less conformal techniques (Eisbruch et al., 1996, Eisbruch et al., 2001). A third, poorly investigated factor, inter-related with the preceding two (Nederfors, 2000) is the solute components of saliva, which also have numerous additional roles in the oral cavity (Sreebny, 2010). These data show that the majority of early PG saliva composition changes recover at > 12 months after IMRT. Residual xerostomia after IMRT will only be further reduced by analysis of the dose distribution to the gland and the PG function observed. Sparing a particular region of the PGs showed a reduction in the risk of post-RT xerostomia when applying IMRT (van Luijk et al., 2015) alternatively sparing of other SG tissues in addition to the PGs may be necessary.

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Table 1

Patient and treatment demographics

	Number (%)
Patients	26 (100)
Age (years)	
Median (range)	59.6 (43.0-78.2)
Gender	
Male	22 (85)
Female	4 (15)
Primary Tumour	
Nasopharynx	1 (4)
Oropharynx	10 (38)
Larynx	5 (19)
Hypopharynx	5 (19)
Thyroid	3 (12)
Unknown primary	2 (8)
Tumour histology	
Squamous Cell Carcinoma	23 (88)
Medullary thyroid carcinoma	2 (8)
Papillary thyroid carcinoma	1 (4)
Whole PG radiation dose, Gy (Mean$\pm$SD)	
<i>Unstimulated samples</i>	
Prescribed dose	28.2 ($\pm$ 8.3)
EQD2	29.4 ($\pm$ 8.7)
<i>Stimulated samples</i>	
Prescribed dose	29.7 ($\pm$ 8.9)
EQD2	30.9 ($\pm$ 9.3)
Chemotherapy	
No	11 (42)
Yes	15 (58)

EQD2 = equivalent radiation dose in 2 Gy per fraction

Table 2

Summary of the parotid gland saliva samples, collection condition and time-point

		Number of samples (patients)	
Total PG Saliva samples		142 (26)	
<u>Sample collection condition</u>			
Unstimulated		64 (26)	
Stimulated		78 (26)	
		Condition	
Time-point		Unstimulated	Stimulated
	Before IMRT	28 (26)	33 (26)
	3-6 months after IMRT	23 (23)	31 (24)
	12-24 months after IMRT	13 (12)	14 (12)

Table 3

Baseline PG saliva flow rate, inorganic and organic analyte concentration and secretion rate under unstimulated and stimulated conditions (paired samples, n=26).

	Unstimulated	Stimulated	p-value
PG flow rate (mL/min)	0.14 (0.1-0.18) *	0.34 (0.21-0.47) *	<0.001
Concentration			
Proteins (µg/mL, unless specified)			
Total Protein (mg/mL)	1.95 (1.38-2.35)	1.67 (1.3-2.51)	0.86
Secretory Immunoglobulin A	216.2 (125.3-726.7)	162 (104.3-678.5)	0.9
Beta-2-microglobulin	1.25 (0.72-1.56)	1.15 (0.67-1.52)	0.81
Lactoferrin	27.9 (8.6-70.8)	44.5 (10.6-65.9)	0.56
Ions (mmol/L)			
Calcium	1.83 (1.46-2.2) *	1.91 (1.53-2.29) *	0.27
Sodium	2.18 (1.85-3.97)	2.46 (1.65-5.17)	0.34
Potassium	4.23 (3.55-5.75)	4.56 (3.58-5.44)	0.001
Magnesium	0.15 (0.1-0.25)	0.14 (0.1-0.23)	0.61
Phosphorus	6.81 (5.9-9.74)	7.03 (5.71-9.23)	0.32
Secretion rate			
Proteins (µg/min, unless specified)			
Total Protein (mg/min)	0.16 (0.09-0.34)	0.39 (0.2-0.69)	0.004
Secretory Immunoglobulin A	21.9 (10.5-46.6)	51.2 (25.7-111.8)	0.002
Beta-2-microglobulin	0.12 (0.06-0.2)	0.26 (0.09-0.37)	0.002
Lactoferrin	3.05 (0.98-7.03)	9.45 (4.13-20.35)	0.01
Ions (nmol/min)			
Calcium	165.8(74-306.7)	364.3 (158.3-826)	0.002

Median (IQR), except * = mean (95% CI), significant if p<0.05

Table 4a and b

Salivary parameters for stimulated and unstimulated PG saliva, organic (a) and inorganic (b) analyte concentration, secretion rate and salivary flow rate at early and late time points after IMRT (mean±95% CI unless specified).

Parameter	Unstimulated		Stimulated	
	3-6 month (n=23)	12-24 month (n=13)	3-6 month (n=31)	12-24 month (n=14)
a)				
PG Saliva Flow Rate (ml/min) (median, IQR)	0.04* (0.02-0.14)	0.12 (0.04-0.34)	0.15* (0.07-0.31)	0.22 (0.1-0.76)
Total Protein Concentration (mg/ml) (median, IQR)	1.45* (1.16-2.26)	1.73 (1.06-3.17)	1.32 (1.13-1.77)	1.24 (0.91-1.71)
Total Protein Secretion Rate (mg/min)	0.05* (0.02-0.13)	0.21 (0.06-0.44)	0.14* (0.08-0.35)	0.33 (0.1-0.72)
IgA Concentration (µg/ml)	202.6 (149.6-449)	140.1 (89.5-374.7)	153.9 (115.8-334.4)	106.4 (59.3-222.3)
IgA Secretion Rate (µg/min)	11.5 (4.4-26.2)	13.8 (10-51.1)	25.0 (14.9-43.7)	35.1 (17.7-69)
B2M Concentration (µg/ml)	1.63 (0.97-2.67)	1.15 (0.91-3.3)	2.45* (1.73-3.16)	0.99 (0.58-2.26)
B2M Secretion Rate (µg/min)	0.07 (0.03-0.13)	0.15 (0.07-0.32)	0.29 (0.19-0.41)	0.44 (0.21-0.67)
LF Concentration (µg/ml)	152.2* (65.4-239)	109.6* (43.7-175.5)	282.1* (149.6-414.7)	82.7* (51-114.3)
LF Secretion Rate (µg/min)	17.7 (0.31-37.9)	17.2 (0.77-34.9)	28.4* (9.83-47.05)	26.98 (10.68-43.28)
b)				
Parameter	Unstimulated		Stimulated	
	3-6 month (n=23)	12-24 month (n=13)	3-6 month (n=31)	12-24 month (n=14)
Ca²⁺ Concentration (mmol/l)	1.70 (1.49-1.91)	1.79 (1.45-2.13)	1.52* (1.40-1.64)	1.68 (1.37-2.00)
Ca²⁺ Secretion Rate (nmol/min) (median, IOR)	46.76 (23.88-126.51)	198.13 (48.74-424.93)	120.53* (59.75-226.59)	368.67 (88.16-891.44)
Phosphate Concentration (mmol/l)	5.28* (4.46-6.09)	5.44* (4.50-6.38)	4.39* (3.85-4.94)	4.72* (3.76-5.67)

Parameter	Unstimulated		Stimulated	
	3-6 month (n=23)	12-24 month (n=13)	3-6 month (n=31)	12-24 month (n=14)
Na⁺ Concentration (mmol/l) (median, IQR)	2.45 (1.66-3.22)	2.45 (1.56-2.74)	3.03 (1.84-6.24)	2.45 (1.74-2.99)
Mg²⁺ Concentration (mmol/l) (median, IQR)	0.13 (0.08-0.19)	0.09 (0.06-0.12)	0.09* (0.07-0.14)	0.08* (0.05-0.11)
K⁺ Concentration (mmol/l)	4.63* (4.26-5.00)	3.75 (3.13-4.38)	4.51 (4.2-4.82)	3.60* (3.01-4.19)

* = $p < 0.025$, PG = parotid gland, IgA = Immunoglobulin A, B2M = Beta-2-microglobulin, LF = Lactoferrin, Ca²⁺ = Calcium, Na⁺ = Sodium, Mg²⁺ = Magnesium, K⁺ = Potassium

Table 5a and b

Summary table of the significant changes in PG saliva analytes, for samples collected under unstimulated (a) and stimulated conditions (b), at the early and late time-points after IMRT

a)

Parameter	3 to 6 months	12 to 24 months
Saliva flow rate	↓	#
TP concentration	↓	#
TP secretion rate	↓	#
sIgA concentration	#	#
sIgA secretion rate	#	#
LF concentration	↑	↑
LF secretion rate	#	#
B ₂ M concentration	#	#
B ₂ M secretion rate	#	#
Ca ²⁺ concentration	#	#
Ca ²⁺ secretion rate	#	#
Na ²⁺ concentration	#	#
Mg ²⁺ concentration	#	#
K ⁺ concentration	↑	#
Phosphate concentration	↓	↓

b)

Parameter	3 to 6 months	12 to 24 months
Saliva flow rate	↓	#
TP concentration	#	#
TP secretion rate	↓	#
sIgA concentration	#	#
sIgA secretion rate	#	#
LF concentration	↑	↑
LF secretion rate	↑	#
B ₂ M concentration	↑	#
B ₂ M secretion rate	#	#
Ca ²⁺ concentration	↓	#

b)

Parameter	3 to 6 months	12 to 24 months
Ca ²⁺ secretion rate	↓	#
Na ⁺ concentration	#	#
Mg ²⁺ concentration	↓	↓
K ⁺ concentration	#	↓
Phosphate concentration	↓	↓

= no significant difference from baseline. TP = total protein, sIgA = secretory immunoglobulin A, LF = lactoferrin, B₂M = beta-2-microglobulin,

Ca²⁺ = calcium, Na⁺ = sodium, Mg²⁺ = magnesium, K⁺ = potassium

Table 6a

Summary table of literature search for studies reporting the composition changes for individual ductal saliva samples after radiotherapy

Author	No. of Patients (PG), RT technique/design	Saliva specimen	Analytes	SG dosimetry	Composition change after radiotherapy (concentration unless specified, significant only if specified)			
					0-3 m	3-6 m	>6-12 m	>12 m
Funegård <i>et al</i> [20]	N = 25 (16) (9 excluded as no pre-RT flow) 2D-RT, prospective	PG (stim)	Hexosamine, SPO, Sialic acid, TP, Amylase, slgA, BAGP, Ca ²⁺ , Na ⁺ , K ⁺	~40-52 Gy both PGs	↓PG flow, ↑TP, ↑SPO sec., ↑K ⁺ , ↓Hexosamine, ↑Sialic acid (n=15 PGs)	↓PG flow, ↑TP, ↑K ⁺ , ↑slgA, ↑SPO sec. (n=15 PGs)	↓PG flow, ↓Amylase, ↓Na ⁺ , ↓Ca ²⁺ (n=10 PGs)	All n.s. (n=12 PGs)
Valdez <i>et al</i> [21]	N = 50 (~25) 2D-RT, cross-sectional	PG + SM/SL (stim)	TP, LF, LZ, Na ⁺ , Cl ⁻ , K ⁺	~60.3 Gy Both PG fully/partly irradiated (n = 49) SM/SL fully/partly irradiated (n=48)	#	#	#	↓PG + SM/SL flow ↑PG + SM/SL Cl ⁻ , ↑PG LF and LZ ↑PG LF sec.
Dijkema <i>et al</i> [29]	N = 29 (n/a) Not Specified, cross-sectional	SM/SL (stim)	MUC5b, TP	Not specified	#	#	n.s.	#

m = months, stim/unstim = stimulated/unstimulated saliva collections, SG = Salivary Gland, PG = Parotid Gland, SM/SL = Submandibular/Sublingual Gland, TP = Total protein, slgA = secretory immunoglobulin A, LF = Lactoferrin, SPO = salivary peroxidase, BAGP = bacterial aggregating glycoprotein, LZ = lysozyme, Muc5b = Mucin 5b, Flow = flow rate, sec. = secretion rate, n.s. = non-significant, # = no samples

Table 6b

Summary table of literature search for studies reporting the composition changes for whole mouth saliva samples after radiotherapy

Author	Patients, RT Technique/design	Saliva specimen	Analytes	SG dosimetry	Composition change after radiotherapy (concentration unless specified, significant only if specified)			
					0-3 m	3-6 m	>6-12 m	>12 m
Randall <i>et al</i> [22]	N = 37 IMRT, prospective	Stim	Ca ²⁺ , MUC5b	CL – 34.8 Gy IL – 43.9 Gy	↓WMS flow (n=20)	#	#	#
Tiwana <i>et al</i> [23]	N = 53 2D-RT, prospective	Stim	TP, pH, Amylase, Na ⁺ , Cl ⁻ , K ⁺	~60-70 Gy All major SGs	↓WMS flow	↓WMS flow, ↓K ⁺	#	#
Makkonen <i>et al</i> [24]	N = 11 2D-RT, prospective	Stim	TP, LF, LZ, Amylase, MPO, SPO, Albumin	~60-70 Gy All major SGs	#	↓WMS flow, ↓Amylase, ↓Amylase sec.(n=5)	#	#
Brown <i>et al</i> [25]	N = 30 2D-RT, prospective	Stim	TP, LZ, IgG, IgA, albumin	>50 Gy All major SGs	↓WMS flow, ↓TP sec., ↓IgA sec.	#	#	#
De Barros-Pontes <i>et al</i> [26]	N = 21 Not Specified, prospective	Stim	TP, Amylase, pH	Not specified	↓WMS flow, ↓Amylase, ↓pH	#	#	#
Christensen <i>et al</i> [36]	N = 21 Not Specified, prospective	Stim	TP, EGF, Amylase, Haptocorrin	Not specified	↓EGF, ↓Amylase, ↑TP, ↑Haptocorrin,	#	#	#
Ben-Aryeh <i>et al</i> [27]	N = 20 2D-RT, prospective	Stim	TP, pH, Amylase, Na ⁺ , K ⁺ , Ca ²⁺ , Mg ²⁺ , PO ₄ ²⁻	~30 Gy, median dose to BL PG	↓WMS flow, ↑Na ⁺	#	#	#
Hannig <i>et al</i> [37]	N = 10 2D-RT, cross-sectional	Unstim	Histatin-5, bPRP, aPRP, statherin, cystatin, α-amylase	~60 Gy All major SGs	#	#	↓WMS flow, ↓aPRP	#
Almstahl <i>et al</i> [28]	N = 9 Not Specified, cross-sectional	Stim + unstim	HCO ₃ ⁻ , Na ⁺ , K ⁺ , Ca ²⁺ , PO ₄ ²⁻	Not specified	#	#	#	↓HCO ₃ ⁻ , ↑K ⁺ , ↑Ca ²⁺ , ↓HCO ₃ ⁻ , ↓K ⁺ , ↑Ca ²⁺ and ↓PO ₄ ²⁻ sec

m = months, stim/unstim = stimulated/unstimulated saliva collections, SG = Salivary Gland, WMS = Whole mouth saliva, BLPG = Bilateral parotid gland, CL = Contralateral PG mean radiation dose, IL = Ipsilateral PG mean radiation dose, TP = Total protein, IgA/G = Immunoglobulin A or G, LF = Lactoferrin, SPO = Salivary peroxidase, MPO = Myeloperoxidase, LZ = Lysozyme, EGF = epidermal growth factor, a/b-PRP = acidic/basic-proline rich protein, Muc5b = mucin 5b, Flow = flow rate, sec. = secretion rate, # = no samples