A Longitudinal Metagenomic Comparative Analysis of Oral Microbiome Shifts in Patients Receiving Proton Radiation versus Photon Radiation for Head and Neck Cancer

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Abstract

Introduction: Due to the radiation-sparing effects on salivary gland acini, changes in the composition of the oral microbiome may be a driver for improved outcomes in patients receiving proton radiation, with potentially worse outcomes in patients exposed to photon radiation therapy. To date, a head-to-head comparison of oral microbiome changes at a metagenomic level with longitudinal sampling has yet to be performed in these patient cohorts. **Methods and Materials:** To comparatively analyze oral microbiome shifts during head and neck radiation therapy, a prospective pilot cohort study was performed at the Maryland Proton Treatment Center and the University of Maryland Marlene and Stewart Greenebaum Comprehensive Cancer Center. A longitudinal metagenomic comparative analysis of oral microbiome shifts was performed at three time points (pre-radiation, during radiation, and immediately post-radiation). Head and neck cancer patients receiving proton radiation ($n = 4$) were compared to photon radiation ($n = 4$). Additional control groups included healthy age- and sex-matched controls ($n = 5$), head and neck cancer patients who never received radiation therapy ($n = 8$), and patients with oral inflammatory disease ($n = 3$). **Results:** Photon therapy patients presented with lower microbial alpha diversity at all timepoints, and there was a trend towards reduced species richness as compared with proton

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therapy. Healthy controls and proton patients exhibited overall higher and similar diversity. A more dysbiotic state was observed in patients receiving photon therapy as compared to proton therapy, in which oral microbial homeostasis was maintained. Mucositis was observed in 3/4 photon patients and was not observed in any proton patients during radiation therapy. The bacterial de novo pyrimidine biosynthesis pathway and the nitrate reduction V pathway were comparatively higher following photon exposure. These functional changes in bacterial metabolism may suggest that photon exposure produces a more permissive environment for the proliferation of pathogenic bacteria. **Conclusion:** Oral microbiome dysbiosis in patients receiving photon radiation may be associated with increased mucositis occurrence. Proton radiation therapy for head and neck cancer demonstrates a safer side effect profile in terms of oral complications, oral microbiome dysbiosis, and functional metabolic status.

Keywords: Head and Neck Cancer, metagenome, oral microbiome, radiation therapy

Introduction

The current standard nonsurgical management of nonmetastatic head and neck carcinoma is definitive or curative-intent radiotherapy with or without chemotherapy. Despite modern techniques such as intensity-modulated photon radiation therapy (IMRT), head and neck cancer patients frequently experience significant acute and chronic oral toxicities. Curative-intent radiotherapy with intensity-modulated proton therapy (IMPT) for nonmetastatic oropharyngeal carcinoma has shown promising clinical results and improved patient-reported outcomes.[1-3] IMPT has been associated with a significantly reduced acute toxicity burden and more favorable locoregional recurrence rates as compared with IMRT.[1] This is largely attributed to the advantage of a reduced delivery of radiation doses to collaterally healthy tissues.

The oral microbiome plays a significant role in oral complications secondary to radiotherapy for head and neck cancer and may affect overall cancer outcomes. It has been established that IMRT for head and neck cancer can lead to dysbiosis of the oral microbiome.[4,5] Consequently, an oral dysbiotic state and direct insult from the effects of

radiation therapy may increase the risk of highly morbid radiation-induced iatrogenic conditions such as oral mucositis and radiation-induced salivary hypofunction and xerostomia. Resultant shifts in the diversity and species richness of the oral microbiome composition as a consequence of irradiation could potentially allow for the emergence of putative pathogens, prolong oral mucositis, prolong acute and long-term oral toxicities, and predispose to oral infections with a risk of life-threatening bacteremia, often necessitating antimicrobials and prolonging in-patient hospitalization.

Photons do not have physical mass and therefore can pass beyond tumoral tissue, resulting in collateral damage to adjacent normal tissue and salivary glands. Protons, on the other hand, have physical mass and stop at a certain depth inside the tumoral tissue. Without the exit dose, protons improve the sparing of surrounding organs and normal tissues, which may result in fewer side effects because less healthy tissue is affected. Due to the radiation-sparing effects on salivary gland acini and oral tissues, changes in the composition of the oral microbiome may be a driver for improved outcomes in patients receiving proton radiation.

However, to date, a head-to-head comparison of the oral microbiome or the functional metabolic status at a metagenomic level with longitudinal sampling before, during, and after radiation therapy has yet to be performed in patients receiving IMRT or IMPT for head and neck cancer. We hypothesize that bacterial and functional shifts in the microbiota represent early driver events that correlate with the severity of oral candidiasis and mucositis.

Materials and Methods

Study design

A prospective pilot cohort of 8 patients were recruited at the Maryland Proton Treatment Center and the University of Maryland Marlene and Stewart Greenebaum Comprehensive Cancer Center [Table 1]. The University of Maryland Baltimore Institutional Review Board approved this study (HP-00087613), all patients signed informed consent,

and all study procedures were in accordance with the Declaration of Helsinki. Head and neck cancer patients receiving proton radiation (*n* = 4) were compared to photon radiation (*n* = 4). As illustrated in Table 1, patients in these two cohorts had similar matching in terms of age, sex, histology, and tumoral site. Additional control groups included (1) patients with oral or oropharyngeal squamous cell carcinoma who have never received radiation therapy to the head and neck (*n* = 8), (2) oral inflammatory mucosal disease (oral lichen planus) (*n* = 3), and (3) age- and sex-matched healthy individuals without any prior history of radiation therapy or oral inflammatory mucosal disease (*n* = 5). The following eligibility criteria were used to enroll subjects for this pilot study:

Inclusion criteria

(a). Study subjects (>18 years old) already planned for treatment to receive radiotherapy (IMRT,

Table 1: Patient characteristics of study cohorts

Patient characteristic	IMRT (*n*=4)	IMPT (*n*=4)
Mean age (range)	67 years (60–72)	62 years (54–77)
Male sex (percentage)	4 (100)	4 (100)
Caucasian race	3 (75)	4 (100)
Current cigarette smoking	3 (75)	2 (50)
Chemotherapy	2 (50)	2 (50)
Surgery	2 (50)	0 (0)
Histology	4 (100) SCC	4 (100) SCC
Tumor site	1 (25) Glossotonsillar 1 (25) Base of tongue 1 (25) Tonsil/Base of tongue 1 (25) Supraglottic	1 (25) Glossotonsillar 3 (75) Tonsil
HPV status	2 (50) Positive 2 (50) Not performed	4 (100) Positive
Radiation parameters		
Prescription dose	2 (50) 69.96 Gy 2 (50) 60 Gy	4 (100) 69.96 Gy
Dose/fraction	2 (50) 2.12 Gy 2 (50) 2 Gy	4 (100) 2.12 Gy
1 Fractions/day	4 (100)	4 (100)
Parotid total mean dose (range)	32.09 Gy (26.69–40.9)	23.96 Gy (21.31–29.7)
Oral cavity total mean dose (range)	41.01 Gy (28.87–61.11)	19.59 Gy (15.6–25.43)

SCC: Squamous cell carcinoma; HPV: Human papillomavirus; IMRT: Intensity-modulated photon radiation; IMPT: Intensity modulated proton therapy; Gy: Greys

n = 4 and IMPT, n = 4) for a primary malignant neoplasm (squamous cell carcinoma; TxN + M0-1a) of either oral, oropharyngeal, hypopharyngeal, or laryngeal primary tumor site.

Exclusion criteria

(a). History of HIV or other immunosuppression
(b). Secondary malignant neoplasms or recurrent primary tumors
(c). Prior radiation therapy to the head and neck
(d). Patients with previous salivary gland disease or co-morbidities such as Sjogren's Syndrome or medications known to cause significant fluctuations in salivary function
(e). Recent (within 3 months) antibiotics, current gross dental disease, use of steroid inhalers, or systemic steroids
(f). Patients receiving radiation therapy for hematological malignancies of the head and neck.

Oral mucosal examination

Oral soft tissues were evaluated for acute or chronic oral mucosal complications. The World Health Organization oral mucositis assessment scale was used for mucositis assessment.[6] Oral candidiasis was diagnosed by an oral medicine expert and confirmed by fungal culture. Xerostomia (subjective patient-reported) assessment was completed using the CTCAE scale, and salivary hypofunction (objective measurement) was defined as an unstimulated whole saliva flow rate of ≤0.1 ml/min.[7]

Sample collection

Measurements and saliva samples were obtained by a calibrated clinician and occurred at several longitudinal timepoints that included:
1. Baseline (prior to initiation of radiotherapy),
2. Approximately midway through radiotherapy,
3. At the end of radiotherapy,

Saliva samples were collected over a 5-min time period. The time and volume of serous and mucous fractions of collected saliva will be recorded to assess changes in the quality and quantity of saliva production.

DNA extraction

The study participants' saliva samples were collected using the Swab Collection and DNA Preservation System (Norgen Biotek Corp., Thorold, Canada). Genomic DNA was extracted using the Quick-DNA Fungal/Bacterial Microprep Kit (Zymo ResearchCorp., Irvine, CA, USA) according to the manufacturer's recommendations. Both positive and negative controls (Zymo Research Corp., Irvine, CA, USA) were included in the DNA extraction process. DNA concentrations in the samples were determined with the Bioanalyzer 2100 DNA 1000 chip (Agilent, Santa Clara, CA, USA).

Shotgun metagenomics

Shotgun metagenomic sequence libraries were constructed from the DNA extracts using Illumina Nextera XT Flex kits according to manufacturer recommendations and then sequenced on an Illumina HiSeq 4000 platform (150 bp paired-end mode) at the Genomic Resource Center at the University of Maryland School of Medicine. Each sample was uniquely barcoded in each HiSeq 4000 lane, yielding an average of 40 million read pairs for each sample. The sequencing data is publicly available (https://www.ncbi.nlm.nih.gov/sra/PRJNA997379).

Bioinformatics analysis

Quality control of each metagenome was performed using tools from the BBMap software package. Taxonomic and functional profiling was performed using tools from the BioBakery 3 suite.

Statistical analysis

Statistical analyses were performed using R (version 3.6.0). The phyloseq R package was used for analysis of the microbial community data. Longitudinal comparisons of alpha diversity metrics, differences in microbial taxa, and metabolic pathways

were assessed using univariable and multivariable linear regressions. Pairwise comparisons were performed with a post hoc Tukey HSD test with the FDR *P* value adjustment set at level 0.05.

Results

Longitudinal shifts in the oral microbiome

Relative abundances of oral microbial taxa between IMRT and IMPT recipients were measured pre-

radiation exposure, during radiation treatment, and immediately post-radiation exposure. Veillonella, Streptococcus, Neisseria, and Actinomyces (genera known to be linked to oral health status[8]) were more abundant in patients during IMPT, whereas Lymphocryptovirus, and Lactobacillus, Capnocytophaga (genera known to be linked to disease[9-12]) were more abundant in patients during IMRT [Figure 1a]. Capnocytophaga has been associated with radiation therapy, mucositis
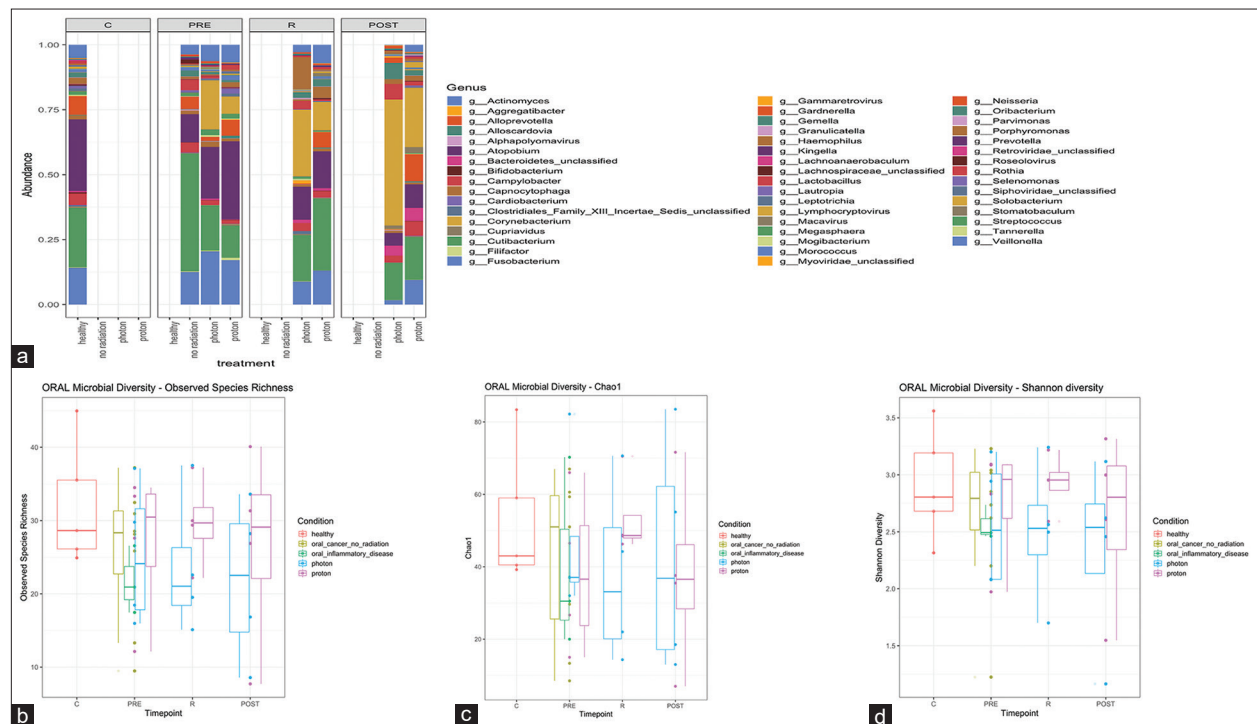

Figure 1: (a) Oral microbial taxa relative abundance differences between healthy, no radiation, photon, and proton radiation treatment recipients. Relative abundances of oral microbial taxa between photon and proton radiation recipients were measured pre-radiation exposure (PRE), during radiation treatment (r), and post-radiation exposure (POST). Veillonella, Streptococcus, Neisseria, and Actinomyces were more abundant in patients during proton radiation recipients, whereas Lymphocryptovirus, Lactobacillus, Capnocytophaga were the taxa that were more abundant in patients during photon radiation recipients. (b) Oral alpha diversity (observed species richness) for healthy, no radiation, oral inflammatory, photon, and proton radiation treatment recipients. Alpha diversity between photon and proton radiation recipients. Age and sex matched healthy controls (orange), head and neck cancer control without radiation therapy (lime green), oral inflammatory disease without radiation therapy (green), conventional photon radiation therapy (blue), proton radiation therapy (pink). C: Healthy controls, PRE: Baseline sample collection pre-radiation, R: Sample collection during radiation, POST: Sample collection immediately following the completion of radiation. No significant difference was observed between the treatments and timepoints (*P* > 0.05). (c) Oral alpha diversity (Chao1) for healthy, no radiation, oral inflammatory, photon, and proton radiation treatment recipients. Alpha diversity between photon and proton radiation recipients was measured PRE, during radiation treatment (r), and POST. No significant difference was observed between the treatments and timepoints (*P* > 0.05). (d) Shannon alpha diversity for healthy, no radiation, oral inflammatory, photon, and proton radiation treatment recipients. No significant shifts in diversity were observed across timepoints: PRE, R, POST

occurrence and severity, and is implicated in serious bacteremia; notably, this species was found to be more abundant in IMRT patients and not IMPT patients in our study.[9,13-15]

Photon therapy patients presented with lower microbial alpha diversity at all timepoints, and there was a non-statistically significant ($P > 0.05$) trend towards reduced species richness as compared with proton therapy [Figure 1b-d]. Healthy controls and proton patients exhibited overall higher and similar diversity. Therefore, a more dysbiotic state is observed in patients receiving photon therapy as compared to proton therapy, in which oral microbial homeostasis is maintained.

Functional changes in bacterial metabolism

The pyrimidine deoxyribonucleotides de novo biosynthesis superpathway (PWY-7211) was comparatively higher after photon exposure [Figure 2a], which could play a role in increasing bacterial proliferation and growth.[16,17] It is postulated that during photon radiation therapy, this superpathway is upregulated in response to the DNA damage that occurs to the oral and gut microbiota. Since photon therapy appears to be more damaging to the surrounding tissues and altering the microbiome, it could be speculated that the upregulation of the nitrate reduction V pathway [Figure 2b] provides compensatory repair of damaged bacterial cellular components.

Oral complications

Mucositis was observed in 3/4 photon patients during radiation therapy, which persisted to the end of therapy [Figure 3]. Grade 2 mucositis was present in 2/3 patients, and grade 3 mucositis was present in 1/3 patients. No mucositis was observed in any of the proton patients during radiation therapy but was observed in 1/4 proton patients at the end of therapy, with a score of 2. Oral candidiasis was observed in 2 out of 4 photon patients and in 1 out of 4 proton patients during radiation therapy. Oral candidiasis persisted to the end of therapy in two photon patients. Xerostomia occurred in all patients except for one proton patient. A CTCAE xerostomia score of 2 was observed in 3/4 photon patients. All proton patients had a CTCAE score of 1. Salivary hypofunction only occurred in one photon patient at the end of therapy.

Discussion

According to the National Association for Proton Therapy, there are 43 proton therapy centers in
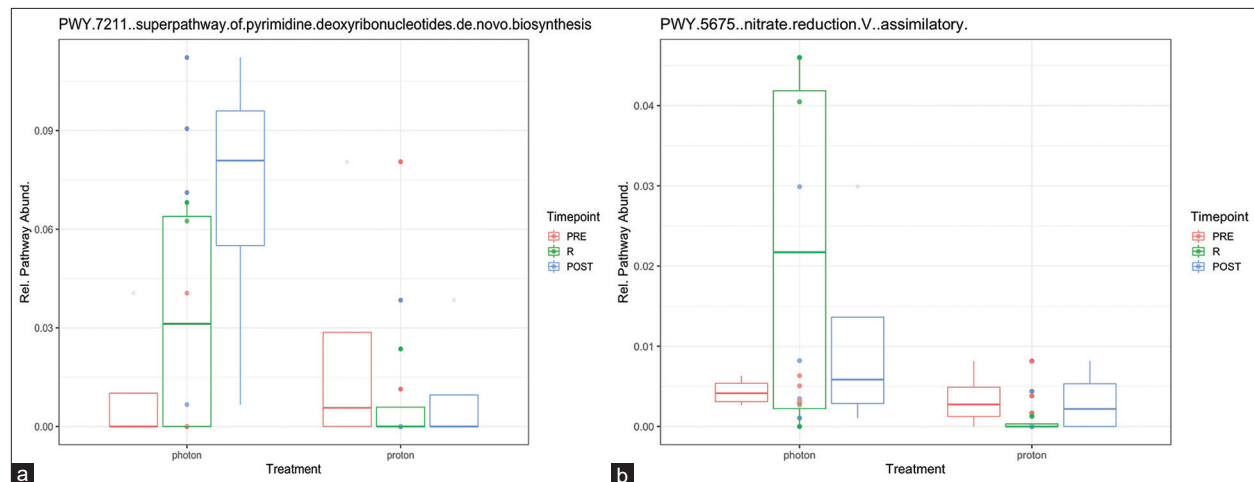

Figure 2: (a) Pyrimidine deoxyribonucleotides de novo biosynthesis superpathway (PWY-7211). PWY-7211 was comparatively higher after photon exposure. No significant difference was observed between the treatments and timepoints ($P > 0.05$). (b) Nitrate reduction V pathway. The nitrate reduction pathway was upregulation during photon treatment. No significant difference was observed between the treatments and timepoints ($P > 0.05$)
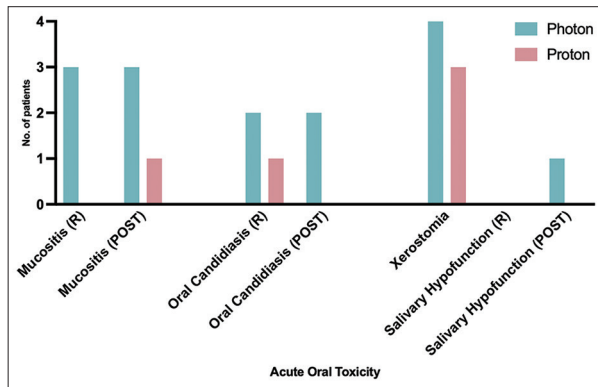
Figure 3: Oral complications and clinical correlates. Oral complications were determined by a board-certified oral medicine specialist (TFM), calibrated for the assessment of oral mucositis, salivary hypofunction, and fungal overgrowth with the assistance of a PhD mycologist (MAJ-R) that assessed all fungal characterization. The World Health Organization oral mucositis assessment scale was used for oral mucositis determination. Each assessment was made prior to the onset of therapy, midpoint of therapy, and at the endpoint. Saliva collections were timed to assess volumetric salivary hypofunction, coupled with culturing to determine the presence of fungal species, determined to be either carriage or infection. Mucositis was observed in 3 out 4 photon patients during radiation therapy (r) and mucositis persisted to the end of therapy (POST). No mucositis was observed in any of the proton patients during radiation therapy (r) but mucositis was observed in 1/4 patients at the end of therapy (POST). Oral candidiasis was observed in 2 out 4 photon patients and in 1 out 4 proton patients during radiation therapy (r). Oral candidiasis persisted to the end of therapy (POST) in 2 photon patients. Xerostomia occurred in all patients except for 1 proton patient. Salivary hypofunction only occurred in one photon patient at the end of therapy (POST)

the United States (U.S.). The U.S. Food and Drug Administration approved proton beam therapy in 1988, and it is currently indicated for the treatment of various malignancies, including but not limited to unresectable malignancies, pediatric malignancies, malignancies of the skull base or adjacent to vital organs, prostate cancer, and head and neck cancer. In fact, because IMPT has a Bragg peak that limits dose distal to the targeted area, it is recommended in the base of skull and nasopharyngeal cancers due to the decreased risk of neuronal tissue toxicity. IMPT use in oropharyngeal carcinoma has

shown promising clinical results and improved patient-reported outcomes;[1-3,18] specifically, recent data suggests a significantly reduced acute toxicity burden and more favorable locoregional recurrence rates as compared with IMRT.[1-3,19-25]

The potential contribution of oral microbiome shifts to oral complications secondary to radiotherapy for head and neck cancer that may affect overall cancer outcomes has attracted significant attention. In fact, it has been shown that IMRT for head and neck cancer can lead to dysbiosis of the oral microbiome.[4,5,26] Consequently, an oral dysbiotic state and direct insult from the effects of radiation therapy may increase the risk of highly morbid radiation-induced iatrogenic conditions such as oral mucositis and radiation-induced salivary hypofunction. Resultant shifts in the diversity and species richness of the oral microbiome composition as a consequence of irradiation could potentially allow for the emergence of putative pathogens. In turn, this may prolong oral mucositis, prolong acute and long-term oral toxicities, and predispose to oral infections with a risk of life-threatening bacteremia, often necessitating antimicrobials and prolonging inpatient hospitalization.

Importantly, oral microbiome shifts have not been previously studied in patients receiving IMRT or IMPT at a metagenomic level, which was conducted in our study. In fact, only 16S rRNA sequencing has been performed, but only in patients receiving IMRT.[5] Preliminary results from this prospective observational pilot study suggest that proton radiation therapy for head and neck cancer may demonstrate a safer side effect profile in terms of oral complications, oral microbiome dysbiosis, and functional metabolic status. The main limitations of this exploratory pilot study were the relatively small sample size, the lack of matching of radiation doses, and the fact that analysis at a metatranscriptomic level was not performed. Recent microbiological studies have elucidated the functional roles of specific bacteria in a plethora of different oral diseases via the metatranscriptome[27,28] and therefore, this approach will complement the metagenomic work

carried out in this pilot study. As the microbiome is a complex system that involves activities and interactions between its microorganisms and their host, the metatranscriptome is critical to better understanding which microorganisms are passive or proactive in the pathogenesis of oral complications. Therefore, future integration of metagenomic and metatranscriptomic data will shed important insights on host-microbial interactions.

We expect to validate this hypothesis in a well-designed, larger clinical study and mechanistically demonstrate that changes in the composition of the oral microbiome may be a driver for worse outcomes in patients receiving IMRT and improved outcomes in IMPT patients. Additionally, multicenter prospective longitudinal studies with strict matching of cohorts utilizing a metagenomic approach are warranted to confirm the microbiome dysbiosis and functional changes observed in this pilot study.

Acknowledgment

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Author Contributions