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PhD Thesis

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In the Era of HPV-Positive Oropharyngeal Squamous Cell Carcinoma: Patient Demographics, Prognosis, and Mesenchymal Stem Cell Therapy for Post-Treatment Side Effects

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Carlander AF, Bendtsen SK, Rasmussen JH, Jakobsen KK, Garset-Zamani M, Grønhøj C, Friborg J, Hutcheson K, Johnson FM, Fuller CD, Moreno AC, Babarinde T, Gross ND, Myers JN, von Buchwald C. "Clinical and prognostic differences in oropharyngeal squamous cell carcinoma in USA and Denmark, two HPV high-prevalence areas." *Eur J Cancer*. 2024 May;202:113983. doi: 10.1016/j.ejca.2024.113983. Epub 2024 Mar 2. PMID: 38452723; PMCID: PMC11357839.

Paper II:

Carlander AF, Gundestrup AK, Jansson PM, Follin B, Hoeeg C, Kousholt BS, Larsen RT, Jakobsen KK, Rimborg S, Fischer-Nielsen A, Grønhøj C, Buchwald CV, Lynggaard CD. "Mesenchymal Stromal/Stem Cell Therapy Improves Salivary Flow Rate in Radiation-Induced Salivary Gland Hypofunction in Preclinical in vivo Models: A Systematic Review and Meta-Analysis." *Stem Cell Rev Rep*. 2024 May;20(4):1078-1092. doi: 10.1007/s12015-024-10700-y. Epub 2024 Mar 2. PMID: 38430363; PMCID: PMC11087340.

Paper III:

Carlander AF, Jakobsen KK, Todsen T, Paaske N, Madsen AKØ, Bendtsen S, Kastrup J, Friborg J, Lynggaard CD, Hauge AW, Christensen R, Grønhøj C, von Buchwald C. "Long-term effectiveness and safety of mesenchymal stromal cell therapy for radiation-induced hyposalivation in head and neck cancer survivors: A randomised, phase-2, trial." *In review*.

Paper IV:

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Disclosures

Rigshospitalet, Copenhagen University Hospital and University of Copenhagen owns a patent for “Stem cell therapy for patients with salivary gland dysfunction”, PCT/EP2020/053878 for which Charlotte Lynggaard, Christian Grønhøj, and Christian von Buchwald are co-inventors. Jens Kastrup, Annette Ekblond, and Mandana Haack-Sørensen hold a patent for “Stem cell therapy based on adipose-derived stem cells”, WO2017068140.

Abbreviations

ASC	adipose-derived mesenchymal stem cell
AJCC	American Joint Committee on Cancer
ARRIVE	Animal Research: Reporting of In Vivo Experiments Guidelines
ASCO	American Society of Oncology
BSC	bone marrow-derived mesenchymal stem cell
CDK	cyclin D-cyclin dependent kinase
CRT	chemoradiotherapy
CT	computed tomography
ctHPV DNA	circulating tumor HPV DNA
DAHANCA	the Danish Head and Neck Cancer Group
DNA	deoxyribonucleic acid
DSA	donor specific antibodies
DSMO	dimethyl sulfoxide
EORCT QLQ-H&N35	European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire - Head and Neck 35
EORCT HNDR	EORCT domains for dry mouth
EORCT HNSS	EORCT domains for sticky saliva
EOCRT HNSW	EORCT domains for swallowing
FEES	fiberoptic endoscopic evaluation of swallowing
G1 phase	growth 1 phase
GCP	Good Clinical Practice
Gy	gray
HLA	human leukocyte antigen
HPV	human papillomavirus
HPV+	human papillomavirus-positive
HPV-	human papillomavirus-negative
HR	hazard ratio
IHC	immunohistochemistry
IMRT	intensity modulated radiotherapy
ISH	in situ hybridization
MALT	mucosa-associated lymphoid tissue

MBS	modified barium swallowing
MeSH	medical subject headings
MDT	multidisciplinary team
MHC	major histocompatibility complex
MSC	mesenchymal stem cell
MRI	magnetic resonance imaging;
mRNA	messenger ribonucleic acid
OPSCC	oropharyngeal squamous cell carcinoma
OS	overall survival
p16	$p16^{Ink4a}$-tumor suppressor protein
p16+	p16-positive
p16-	p16-negative
PCR	polymerase chain reaction
PET-CT	positron emission tomography-computed tomography
PROMs	patient-reported outcomes measurements
QoL	quality of life
RFI	recurrence-free interval
RT	radiotherapy
SAE	serious adverse events
SFR	salivary flow rate
SMD	standardized mean difference
S phase	synthesis phase
SYRCLE	Systematic Review Center for Laboratory Animal Experimentation
SWS	stimulated salivary flow rate
The U.S.	the United States of America
TORS	transoral robotic surgery
UTMDACC	University of Texas MD Anderson Cancer Center
UWS	unstimulated salivary flow rate
XQ	xerostomia questionnaire

English summary

The incidence of oropharyngeal squamous cell carcinoma (OPSCC) has been increasing in Denmark and worldwide for the past decades, driven by infection with human papillomavirus (HPV). HPV-positive (HPV+) OPSCC is a distinct clinical and biological entity from OPSCC driven by the traditional risk factors tobacco smoking and alcohol consumption, and, since the early 2000s, a new patient group has emerged. Patients with HPV+ OPSCC are usually younger, have higher socioeconomic status, have less comorbidities, are diagnosed at lower cancer stages and, most importantly, have a better prognosis and survival than patients with HPV-negative (HPV-) OPSCC.

Most head and neck cancer patients, including OPSCC, receive radiotherapy (RT) which is related to substantial acute and late toxicities. As HPV+ OPSCC is associated with a high overall survival, there has been a pursuit to identify a subgroup of patients eligible for de-escalated therapy to reduce treatment-related side effects without compromising survival outcomes. So far, it has not been possible to reliably identify such a subgroup since still 15% of patients with HPV+ OPSCC experience a worse prognosis, with 6% developing distant metastases, most frequently in the lungs. If future de-escalation strategies are to be successful worldwide, across diverse OPSCC populations with distinct healthcare systems, we need to know to what extent different OPSCC cohorts are comparable. Although most patients with OPSCC receive RT worldwide, differences in treatment modalities exist. In Denmark, trans-oral robotic surgery (TORS) has been available since 2013 for low-stage disease (T1-T2/N0-N1) to minimize the side effects from radiation. A broader understanding of the differences in practice patterns and preferred treatment regimens is essential for establishing more uniform guidelines and, hopefully, future implementation of de-escalation strategies.

RT is related to severe long-term toxicities, and the most common side effects are salivary gland damage, hyposalivation, and xerostomia (dry mouth syndrome). Radiation-induced xerostomia and hyposalivation affect oral health; impact chewing, swallowing, and speech; and severely impact the quality of life. Currently, no disease-modifying therapies exist, and only symptomatic treatment is available. Mesenchymal stem cells (MCSs) have immunomodulatory and regenerative abilities and have been investigated as a potential therapeutic agent for radiation-induced salivary gland hypofunction and xerostomia, but the long-term effect in a larger, randomized trial has not been evaluated. The mode of action is not fully understood, but it has been indicated that the salivary proteome composition is significantly altered after MCS therapy, although not restored back to normal. However, this has not yet been validated in randomized trials.

The aim of this thesis was to investigate the interplay of patient specific factors, oncological outcomes, and rehabilitation in head and neck cancer patients in the era of HPV.

Paper I examined the clinical, treatment, and prognostic differences between two high HPV-prevalence OPSCC cohorts from the University of Texas MD Anderson Cancer Center, USA (UTMDACC) and Eastern Denmark. These cohorts represent distinct populations and healthcare systems. In Denmark, we diagnose and treat unselected cancer patients within a universal healthcare system and a focused budget, while UTMDACC, as the highest-ranked cancer center in the USA, operates within an insurance-based system, mostly available for highly-selected, resourceful patients. The study found significant demographic, clinical, and treatment differences, despite both cohorts having a high HPV prevalence. Moreover, the study revealed notable differences in the prognosis, with a higher risk of recurrence in the overall Copenhagen cohort and among advanced stages (III-IV). This highlights the necessity of understanding cohort comparability for optimizing treatment stratification in clinical trials across diverse geographical areas.

Papers II and III examined the safety and effect of transplantation with MSCs for radiation-induced salivary gland hypofunction and xerostomia. **Paper II** was a systematic review and meta-analysis investigating the effect of MSCs in animal models. The study included MSCs from all origins (adipose tissue, bone marrow, and salivary gland tissue) and all administration routes (intraglandular and systemic transplantation). MSCs was associated with a significant increase in salivary flow rate. Remodeling and regenerative effects were observed, highlighting the potential for MSCs as a therapeutic agent for hyposalivation. **Paper III** was a randomized, placebo-controlled clinical trial in humans, investigating the long-term effect of intraglandular transplantation with adipose-derived MSCs (ASCs) for radiation-induced salivary gland hypofunction in head and neck cancer survivors. The study included 120 patients, who were randomized 1:1 to receive either ASCs or placebo in both submandibular glands and were followed for 12 months. The results from the four-month follow-up have previously been published. The primary endpoint was effect of ASCs on unstimulated salivary flow rate (UWS) compared to placebo, while secondary endpoints were effect on the patient-reported outcome measurements of sticky saliva, dry mouth, swallowing, and xerostomia at 12 months. The study revealed no significant objective differences in UWS in patients receiving ASCs compared to placebo. However, there was a significant improvement in the subjective feeling of dry mouth following ASCs compared to placebo at 12 months. Both ASCs and placebo were associated with an increase in UWS, indicating a continuous natural restoration of the salivary glands. Transplantation with ASCs was safe and did not result in serious adverse events, and it was associated with a transient immune response, as most patients who developed donor-specific antibodies at four months exhibited a reduced or resolved response by 12 months. In **Paper IV** we investigated the mode of action following intraglandular

ASC therapy by analyzing the salivary proteome composition at four months in the group receiving ASC therapy compared to placebo. The study did not detect any significant differences in the salivary proteome composition following ASC therapy compared to placebo. However, a non-significant upregulation of salivary proteins upregulated in healthy salivary was seen following ASCs, suggesting a partial repair.

In conclusion, Paper I contributes to a broader understanding of the diversity between a high HPV prevalence OPSCC cohort from UTMDACC with a highly selected OPSCC population from an insurance-based healthcare system and from Eastern Denmark with an unselected OPSCC population from a universal healthcare system, which differences are important to consider for the reproducibility and validation of future de-escalation trials worldwide. Papers II-IV provide novel and translational findings on the potential of ASCs as a treatment for radiation-induced salivary gland hypofunction and xerostomia in head and cancer survivors. While Paper II underlines the preclinical effect of MSC therapy, Papers III-IV shows the long-term effect and mode of action through salivary proteomic profiling of intraglandular ASC therapy in a randomized, phase 2 clinical trial.

Danish summary (dansk resume)

Forekomsten af oropharynx cancer (OPSCC) er steget i Danmark og den vestlige verden over de seneste årtier, på grund af infektion med human papillomavirus (HPV). HPV-positiv (HPV+) OPSCC adskiller sig fra OPSCC forårsaget af de traditionelle risikofaktorer tobaksrygning og alkoholforbrug, og siden begyndelsen af 2000 er en ny patientgruppe derfor opstået. Patienter med HPV+ OPSCC er typisk yngre, har højere socioøkonomisk status, færre komorbiditeter, diagnosticeres i tidligere stadier af kræft og vigtigst af alt, har de en bedre overlevelse sammenlignet med patienter med HPV-negativ (HPV-) OPSCC.

De fleste patienter med hovedhalskræft, herunder OPSCC, modtager strålebehandling, som er forbundet med betydelige akutte og sene bivirkninger. Da patienter med HPV+ OPSCC har en høj overlevelse, har man forsøgt at identificere en undergruppe af patienter, der kan få deeskaleret behandling for at reducere den behandlingsrelaterede toksicitet uden at gå på kompromis med overlevelsen. Indtil videre har det ikke været muligt at identificere en sådan undergruppe, hvilket bl.a. også skyldes at 15% af patienterne med HPV+ OPSCC har en dårlig prognose og 7% får fjernmetastaser, oftest til lungerne. For at fremtidige de-eskaleringsstrategier skal være succesfulde globalt, på tværs af forskellige OPSCC-kohorter og med forskellige sundhedssystemer, er det nødvendigt at forstå i hvilken udstrækning forskellige OPSCC-populationer er sammenlignelige. Selvom de fleste patienter med hovedhalskræft får strålebehandling, er der forskelle i de anvendte behandlingsregimer. I Danmark har transoral robotkirurgi (TORS) været tilgængelig siden 2013 for patienter med lav sygdomsbyrde (T1-T2/N0-N1) for at minimere bivirkninger til strålebehandling. En bredere forståelse af forskellene i praksismønstre og foretrukne behandlingsregimer er afgørende for at etablere mere ensartede retningslinjer og forhåbentlig fremtidig implementering af de-eskaleringsstrategier.

Strålebehandling er forbundet med alvorlige og langsigtede bivirkninger, og den mest almindelige bivirkning er stråle-induceret nedsat spytkirtelfunktion, hyposalivation og tør mund, xerostomi. Stråleinduceret xerostomi og hyposalivation påvirker den orale sundhed, komplicerer tygning, synkning og tale og har en alvorlig indvirkning på patienternes livskvalitet. Aktuelt findes der ingen sygdomsmodificerende behandlingsstrategier, og de eneste tilgængelige behandlinger er symptomatiske. Mesenkymale stamceller (MSCs) har immunmodulerende og regenerative evner og er blevet undersøgt som mulig terapeutisk behandling mod stråleinduceret hyposalivation og xerostomi, men langtidseffekten er endnu ikke blevet undersøgt i større, randomiserede studier. Hvordan de virker, er endnu ikke fuldt belyst, men mindre studier tyder på at sammensætningen af spytproteomet ændrer sig markant efter

behandling med MSCs, dog ikke tilbage til et normalt og rask spytproteom. Dette er dog ikke blevet valideret i større, randomiserede studier.

Formålet med denne afhandling var at undersøge samspillet mellem patientspecifikke faktorer, prognose og rehabilitering hos patienter med hovedhalskræft i HPV-æraen.

Paper I undersøgte kliniske, behandlingsmæssige og prognostiske forskelle mellem to OPSCC-kohorter med høj HPV-prævalens fra The University of Texas MD Anderson Cancer Center, USA (UTMDACC) og Østdanmark. Disse kohorter repræsenterer forskellige populationer og sundhedssystemer: I Danmark diagnosticeres og behandles uselekterede kræftpatienter i et offentligt tilgængeligt sygehusvæsen indenfor visse budgetrammer. Modsat UTMDACC, der som det højest rangerede kræft-hospital i USA opererer i et forsikringsbaseret sundhedssystem, der sædvanligvis kun er tilgængeligt for højt selekterede, ressourcestærke patienter. Studiet fandt betydelige forskelle i demografiske, kliniske og behandlingsmæssige faktorer, på trods af at begge kohorter havde en høj HPV-prævalens. Derudover viste studiet væsentlige forskelle i prognosen, med en højere risiko for recidiv i den østdanske kohorte som helhed og for patienter med avanceret sygdom (stadie III-IV). Resultaterne fremhæver nødvendigheden af at forstå forskellige OPSCC-populationers sammenlignelighed for at optimere behandlingsstratificering i kliniske forsøg på tværs af forskellige geografiske områder.

Paper II og III undersøgte sikkerheden og effekten af transplantation med MSCs som behandling af stråleinduceret hyposalivation og xerostomi. **Paper II** var et systematisk review og meta-analyse, der undersøgte effekten af MSCs i dyremodeller. Studiet omfattede MSCs uanset oprindelse (adipøst væv, knoglemarv og spytkirtelvæv) og alle administrationsveje (intraglandulær og systemisk transplantation). Studiet viste at MSCs var associeret med en signifikant stigning i spytflowraten. Studiet viste også at behandling med MSCs var associeret med strukturelle og regenerative effekter i spytkirtlerne, hvilket understreger deres potentiale som terapeutisk behandling mod hyposalivation. **Paper III** var et humant, randomiseret, placebokontrolleret klinisk studie, der undersøgte den langsigtede effekt af intraglandulær transplantation med fedtderiverede MSCs (ASCs) mod stråleinduceret nedsat spytkirtelfunktion hos patienter med tidligere hovedhalskræft. Studiet inkluderede 120 patienter, der blev randomiseret 1:1 til at modtage enten ASCs eller placebo i begge gll. submandibularis og blev fulgt i 12 måneder. Resultaterne fra fire måneders opfølgningen er allerede udgivet. Det primære endepunkt var effekten af ASCs på ustimuleret spytflowrate (UWS) sammenlignet med placebo, mens sekundære endepunkter var effekt på patientrapporterede oplysninger vedrørende klistret spyt, tør mund, synkefunktion og xerostomi efter 12 måneder.

Studiet viste ingen signifikante objektive forskelle i UWS hos patienter, der modtog ASCs sammenlignet med placebo, men der var en signifikant forbedring af den subjektive følelse af tør mund efter ASCs sammenlignet med placebo. Behandling med både ASCs og placebo var associeret med en stigning i UWS, hvilket indikerer en kontinuerlig naturlig heling af spytkirtlerne. Behandling med ASCs var sikker og var ikke forbundet med alvorlige bivirkninger. Behandlingen var forbundet med et forbigående immunrespons, da de fleste patienter, der udviklede donor-specifikke antistoffer efter fire måneder, havde intet eller et reduceret respons efter 12 måneder. I **Paper IV** undersøgte vi mode of action for ASC-behandling sammenlignet med placebo, ved at undersøge sammensætningen af spytproteomet efter fire måneder. Studiet viste ikke nogen forskelle i sammensætningen af spytproteomet hos dem der fik ASCs sammenlignet med dem der fik placebo. Vi kunne dog vise at flere vigtige spytproteiner, der er opreguleret i rask spyt, også var opreguleret os dem, der fik ASCs sammenlignet med placebo, selvom det ikke var signifikant.

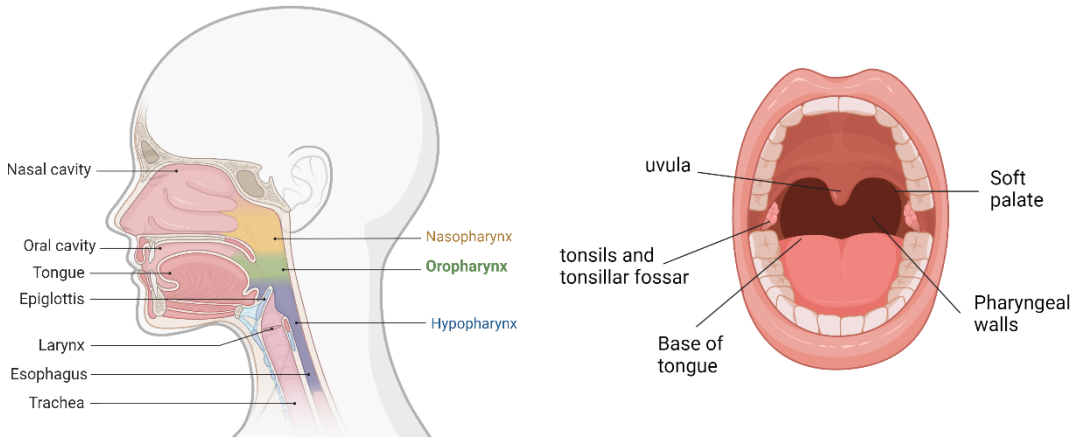
Afslutningsvis, Paper I bidrager til en bredere forståelse af forskellene mellem OPSCC-populationer med høj HPV-prævalens fra UTMDACC med en højt selekteret OPSCC-population i et forsikringsbaseret sundhedssystem og fra Østdanmark med en uselekteret OPSCC-population i et offentligt tilgængeligt sundhedssystem, som er vigtige at overveje for reproducerbarheden og validering af fremtidige deeskaleringsforsøg på tværs af geografiske områder. Paper II-IV giver nye og kliniknære resultater om potentialet for ASCs mod stråleinduceret hyposalivation og xerostomi hos patienter med tidligere hovedhalskræft. Mens Paper II understreger den prækliniske effekt af MSC-behandling, viser Paper III-IV langtidseffekten samt virkemåde ved undersøgelse af sammensætningen af spytproteomet af intraglandulær ASC-behandling i et randomiseret, fase 2 studie.

Introduction

Oropharyngeal cancer

The oropharynx is the middle part of the upper aerodigestive tract and is located posterior the mouth; see **Figure 1**. The anatomy of the oropharynx is complex, and consists of the palatine tonsils and tonsillar fossa, the base of the tongue, soft palate comprising the uvula, and the superior pharyngeal walls[1]. Most often, oropharyngeal cancer arises from the mucosal tissue and the squamous cells as oropharyngeal squamous cell carcinoma (OPSCC)[2,3]. The lingual tonsils are located in the base of the tongue with no midline raphe. The palatine and lingual tonsils consist of specialized lymphoid tissue and are part of the Waldeyer's ring. The palatine tonsils consist of 10-30 invaginated, branched crypts, while the lingual tonsils consist of only one[4]. The crypts are lined with a single layer of stratified squamous, non-keratinized epithelium with mucosa-associated lymphoid tissue (MALT) beneath[4]. The epithelium is directly exposed to the oral cavity, and lymphocytes from the underlying MALT can infiltrate the epithelial layer, creating a close interaction with oral pathogens such as human papillomavirus (HPV)[4].

Figure 1. Anatomy of the oropharynx. The oropharynx is part of the upper aerodigestive tract bounded superiorly by the nasopharynx and inferiorly by the hypopharynx. It comprises the palatine tonsils and tonsillar fossa, the base of the tongue with the lingual tonsils, the superior pharyngeal walls, and the soft palate.



*Created with BioRender.com.

Historically, head and neck cancers, including OPSCC, have been linked to lifestyle risk factors such as tobacco smoking and alcohol consumption, but older age and being male are also associated with head and neck cancer[5,6].

Human papillomavirus and OPSCC

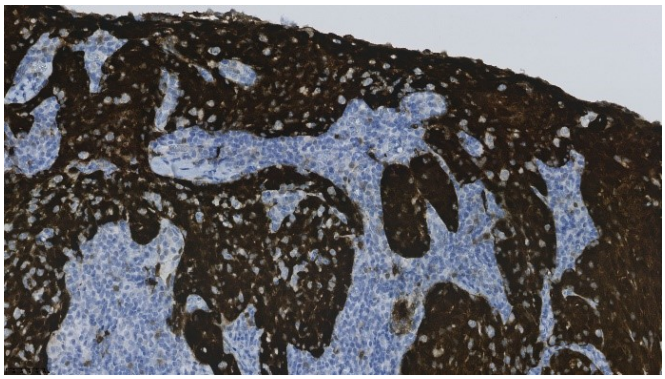
Twenty-five years ago, it became evident that HPV was associated with a distinct entity of head and neck cancers[7–10]. Since 2007, infection with HPV has been a well-established risk factor for OPSCC[11] and the incidence of HPV-positive (HPV+) OPSCC has been increasing worldwide since the early 2000s[12–20]. A rise in the age-adjusted incidence rate pr. 100,00 from 0.9 in the year 2000 to 3.5 in 2020 has been observed in Eastern Denmark[20]. In the same period, the female:male ratio was 1:2.6 for all OPSCC compared to 1:3.1 for HPV+ OPSCC. The burden of OPSCC among men has already surpassed the burden of cervical cancer in the U.S and UK[21]. The prevalence of HPV+ OPSCC varies across different geographical areas[22], and the highest rates are observed in Northern European countries and in the U.S, but Lebanon and South Korea also have a high HPV prevalence[16,18,19,22]. In Eastern Denmark, the HPV prevalence has increased from 46.1% from 2000-2002 to 64.3% from 2018-2020[20], while this is even higher in Sweden[18] and the U.S.[19]. To prevent carcinogenic HPV infections, Denmark implemented a national vaccination program for girls in 2009, aimed at reducing HPV-driven cervical cancer. In 2019, the program was expanded to include boys to increase herd immunity, and with the expectation that it will reduce the incidence of HPV+ OPSCC in the next 30 to 40 years[23].

HPV is the most common sexually transmitted disease, with more than 200 both low-risk and high-risk genotypes[24]. The latter are carcinogenic and responsible for HPV-driven cancers and the most common genotypes are HPV16 and HPV18[25,26]. HPV+ OPSCC is most often caused by chronic infection with HPV16 accounting for over 80% of HPV+ OPSCCs but the high-risk genotypes HPV18, 31, 33, 35, 45, 51, and 58 are observed in relation to HPV+ OPSCC as well[12,16,27]. Transient oral HPV infection is common in the broad population and is most often cleared within one to two years, but some will develop into a persistent infection and a few of these will develop into OPSCC. The natural history of OPSCC development spans over 30-40 years and without precancerous lesions[21].

The gold standard HPV test method is detection of transcriptionally active messenger ribonucleic acid (mRNA) E6/E7, but this is technically demanding and expensive[28]. Therefore, other more cost-effective and accessible techniques are used for evaluating HPV-positivity. Previously, the presence of the protein $p16^{INK4a}$ (p16) assessed by immunohistochemistry (IHC) staining has been used as a surrogate marker for

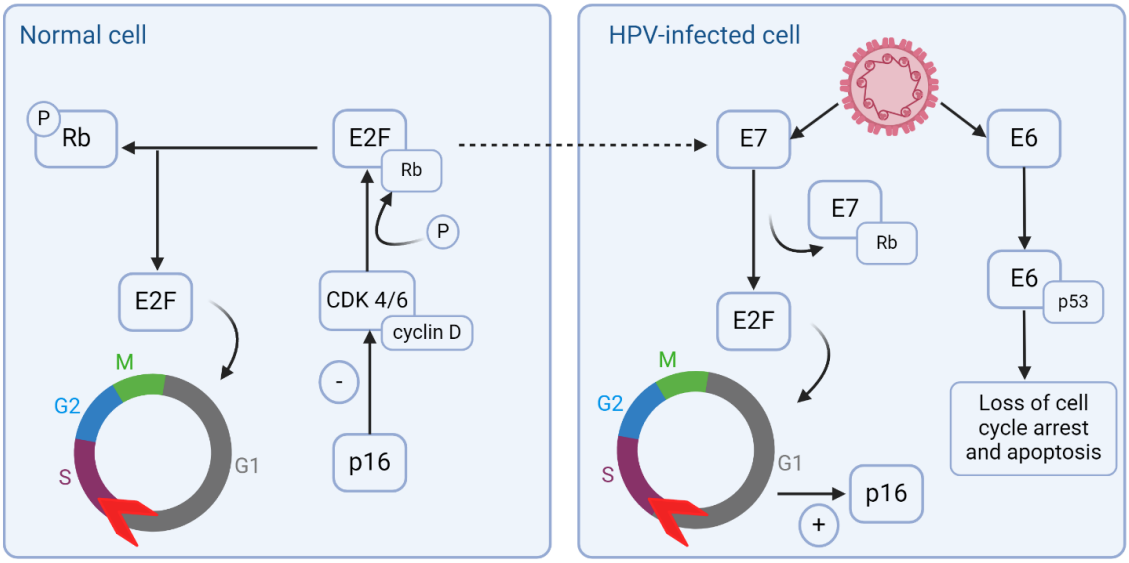
HPV positivity[28–30]. A cut-off of 70% nuclear and cytoplasmic staining is recommended by the American Society of Oncology (ASCO) for classifying p16-positivity; see **Figure 2**[28].

Figure 2. Immunohistochemistry showing p16-positivity. >70% positive nuclear and cytoplasmatic immunostaining for p16 is considered positive. Picture kindly provided by the Department of Pathology, Rigshospitalet, Copenhagen.



The tumor suppressor gene p16 prevents excessive cell growth and division[31]. In normal cells, p16 inhibits cyclin D-cyclin dependent kinase (CDK) 4/6, preventing the phosphorylation of the tumor suppressor protein retinoblastoma (pRb), which in turn hinders the release of the transcription factor E2F into its active state. Consequently, the expression of downstream gene products necessary for the cell to transition from the growth 1 (G1) to the synthesis (S) phase is suppressed. Infection with HPV can lead to upregulation of p16, which is why p16 may be used as a surrogate marker for HPV infection. Integration of HPV deoxyribonucleic acid (DNA) in the host genome leads to the expression of the oncoproteins E6 and E7 which play a crucial role in HPV-induced carcinogenesis[32,33]. E7 displays the E2F from pRb in the E2F-pRB complex, and subsequently E2F becomes active, allowing the cell to enter the S phase. Then p16 is continuously upregulated to inhibit further cell proliferation through feedback mechanisms. Thereafter, E6 binds to the tumor suppressor protein p53, which loses its regulatory function, leading to abnormal cell cycle progression, and inhibits apoptosis. See **Figure 3**.

Figure 3. Regulation pathway of p16 in normal and HPV-infected cells. HPV infection leads to the expression of the viral oncogenes E6 and E7, which inactivate the tumor suppressor proteins p53 and Rb, respectively. This inactivation results in the upregulation of p16, a surrogate biomarker often used to indicate HPV-driven carcinogenesis in OPSCC.



Abbreviations: CDK, cyclin D-cyclin dependent kinase; G, growth; HPV, human papillomavirus; M, mitosis; OPSCC, oropharyngeal squamous cell carcinoma; P, phosphorylation; p16, p16^{INK4a}, Rb, retinoblastoma; S, synthesis.

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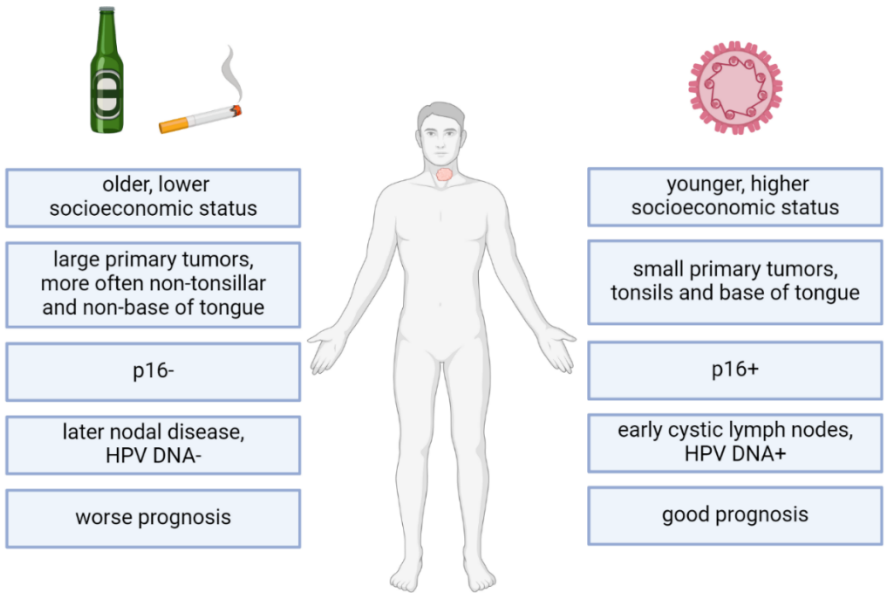
**Figure inspired by Wai et al., *Cells*, 2020[34].

However, the use of p16 as a stand-alone surrogate marker is insufficient, as not all HPV+ tumors show p16 overexpression, and p16 overexpression can occur in tumors not driven by HPV, which impacts the prognosis[12,35]. Other available HPV test methods comprise detection of HPV DNA by polymerase chain reaction (PCR) or in situ hybridization (ISH) and can be used alone or in combination with p16 IHC[28]. These tests enable detection of the presence of HPV DNA in a tumor sample, but they do not confirm whether the HPV DNA is transcriptionally active within the tumor cells. All detection methods have shown overall high sensitivity and specificity[36], but a combination of diagnostic test to assign HPV-status is the most attractive strategy, as double HPV/p16 status is prognostic[12,35,37,38].

The HPV+ OPSCC patient

HPV+ OPSCC exhibit a unique demographic, clinical, genetic, and histopathological profile compared to HPV-negative (HPV-) OPSCC caused by smoking and alcohol consumption. Patients are more often men, with limited history of smoking or alcohol consumption, higher social status, and fewer comorbidities [12,16–18,39–44]. Patients with HPV+ OPSCC are slightly younger than patients with HPV- OPSCC (median age 59 vs. 60)[12], but a rising incidence among elderly and among women has been observed[45,46]. HPV+ OPSCC is most often diagnosed specifically in the palatine tonsils or base of tongue, with patients frequently presenting with a cystic neck mass and small primary tumors[12,47–49]. Importantly, patients with HPV+ OPSCC have a more favorable prognosis[12,42]. The risk factors for acquiring an oral HPV infection are closely related to sexual behavior (e.g., lifetime number of sexual partners and age at sex debut)[50]. See **Figure 4**.

Figure 4. Demographic, clinical, and prognostic differences between the HPV+ and HPV- OPSCC patient. HPV+ OPSCC patients are typically younger, of higher socioeconomic status, and present with small, p16+ tumors specifically located in the palatine tonsils or base of the tongue, often accompanied by cystic nodal metastases, and generally have a favorable overall survival.



Abbreviations: DNA, deoxyribonucleic acid; HPV, human papillomavirus; HPV-, human papilloma virus-negative; HPV+, human papilloma virus-positive; OPSCC, oropharyngeal squamous cell carcinoma; P, phosphorylation; p16, $p16^{INK4a}$, *Created with BioRender.com

In Denmark, patients with HPV+ OPCC have a high five-year overall survival (OS) of 79% compared to 35% in HPV- OPSCC[20]. The long-term prognosis is favorable with 10-year OS rates of 65% for HPV+ OPSCC versus 23% for HPV- OPSCC and a low risk of late recurrences[51,52]. To account for the distinct biology of HPV+OPSCC, which includes nodal metastases but a favorable prognosis, the latest American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) staging system (TNM 8th edition) incorporated p16-positivity to distinguish HPV+ OPSCC from HPV- OPSCC, enhancing prognostication[53]. Currently, HPV DNA testing to confirm transcriptionally active HPV is of debate, but up to 9% of OPSCC patients have discordant p16/HPV status, with p16+/HPV+ patients having a more favorable prognosis compared to p16+/HPV- depending on geographical area and anatomical subsite[12]. To further improve prognostication, combined p16 and HPV testing is important[12].

Diagnosis of OPSCC

Denmark has a universal, publicly-available healthcare system. Cancer diagnosis, treatment, and follow-up regimens are performed within cancer care packages to secure faster and standardized diagnosis and treatment initiation at public university hospitals; see **Figure 5**.[54,55]. Patients with a suspicion of head and neck cancer should be seen within six days, diagnosed within 15 days, and treatment should be initiated within 7-11 days from diagnosis (depending on the treatment regimen given). The total process time is 28 days for surgically-treated patients and 32 days for radio- and/or chemotherapy-treated patients[54].

Figure 5. **Map of Denmark.** The map shows the distribution of the Danish department involved in diagnosis and treatment of head and neck cancer. Red dots represent otorhinolaryngology departments while green dots represent oncological departments.

Red flag symptoms indicative for head and neck cancer including OPSCC, and which should be referred to evaluation within the cancer fast-track program, encompass non-healing wounds or ulcers, visible or palpable tumors, and visualized neck mass but also sore throat, globus sensation, otalgia, persistent pain, or hoarseness > 2 weeks as these could indicate cancer[54,56,57]. The evaluation is conducted at an otorhinolaryngology department in accordance with the Danish Head and Neck Cancer Group (DAHANCA) clinical guidelines[55]. The evaluation comprises a thorough clinical examination and includes both fiberscopy and ultrasound examination. Depending on the patient and tumor-specific characteristics, patients will undergo imaging and a tumor biopsy. Imaging is used to evaluate the extension of the primary tumor (magnetic resonance imaging; MRI), bone involvement (computed tomography; CT) and distant metastasis (positron emission tomography-CT; PET-CT). As HPV+ OPSCC patients often present with a neck mass, fine-needle aspiration can be used to detect HPV DNA within the mass to differentiate metastases from benign disease, guiding the following diagnostic work-up [58].

A definitive diagnosis, including TNM staging, and treatment decision are determined based on a combined assessment of the biopsy, imaging, and clinical examination at a multidisciplinary team (MDT) conference with the patient, an otorhinolaryngologist, and an oncologist[54].

Treatment and follow-up of OPSCC

Three primary treatment modalities for OPSCC are used either alone or in combination: 1) surgery 2) radiotherapy (RT), and 3) chemotherapy, with chemoradiotherapy (CRT) as the standard treatment regimen[55]. The clinical guidelines have not been updated since the introduction of trans-oral robotic surgery (TORS) in Denmark, resulting in the absence of TORS from the current guidelines. The guidelines are currently under revision, which are expected to be implemented in 2026. The choice of treatment depends on several factors such as tumor stage, anatomical location, performance status and comorbidities, functional considerations, and the preferences of both patients and clinicians[55]. Still, most patients undergo curatively-intended, radiation-based primary treatment[59], which in Denmark consists of moderately accelerated RT administered in 33-34 fractions for six days a week with or without concurrent weekly Cisplatin (40 mg/m^2) (CRT) in locoregionally advanced tumors and if tolerated by the patient[55]. The standard total radiation dose equals 66-68 gray (Gy) to the target tissue, while a lower dose of 60 Gy is given to the surrounding tissue and 50 Gy to elective targets[55], minimizing radiation to the healthy, adjacent tissues. Additionally, all patients are offered nimorazole, a hypoxic radiosensitizer[55,60].

Historically, surgery for OPSCC involved extensive, open surgery, but since 2013 TORS has been a treatment option at Rigshospitalet. In May 2024, our department at Rigshospitalet received a new, Da Vinci single port for TORS. TORS is compared with RT as an option for OPSCC patients with low-stage (T1-T2, N0-N1) disease in the randomized clinical trial, DAHANCA34 Trial/QoLATI, (clinicaltrials.gov identification number: NCT04124198) or if primary RT cannot be performed[61]. The primary endpoints in the QoLATI Trial are quality of life (QoL) and swallowing function evaluated by both modified barium swallowing (MBS) and fiberoptic endoscopic evaluation of swallowing (FEES) after TORS, compared to RT, and is nearing its completion with only seven patients awaiting inclusion (+ one year follow-up). TORS is minimally invasive compared to previous open surgical techniques, and it is expected that TORS will avoid RT-related toxicities without compromising the prognosis[62–64]. Primary TORS could pave the way for salvage RT in the event of recurrent disease. However, TORS is still associated with sequelae as reported by the ORATOR trial including early-stage HPV+ OPSCC, though it should be noted that the TORS group underwent neck dissection at the time of surgery or within two weeks[65,66]. Adverse prognostic features such as positive margins or extranodal extension following TORS require adjuvant therapy[67], and to accommodate this, the approach in Denmark is to perform a neck dissection prior to initiation of RT or TORS to minimize the need for multimodality treatment. The question of which treatment modality has a superior sequelae profile is still not fully elucidated and is under investigation in the beforementioned QoLATI trial. When the results from the QoLATI trial are available, it is expected that TORS will find its role in the new treatment guidelines addressing whether TORS is superior to RT in the treatment of T1-T2/N0-N1 OPSCCs. Even though early-stage tumors might be treated with single-modality treatment, some patients with OPSCC require multimodality treatment with increased treatment-related side effects[16]. Neoadjuvant chemotherapy aiming at tumor reduction may enable more patients to receive subsequent TORS[68], and a feasibility study to explore this approach will be initiated at Rigshospitalet in early 2025, comprising patients with advanced T-site (T2-T3/N0-N1) that are not immediately eligible for TORS.

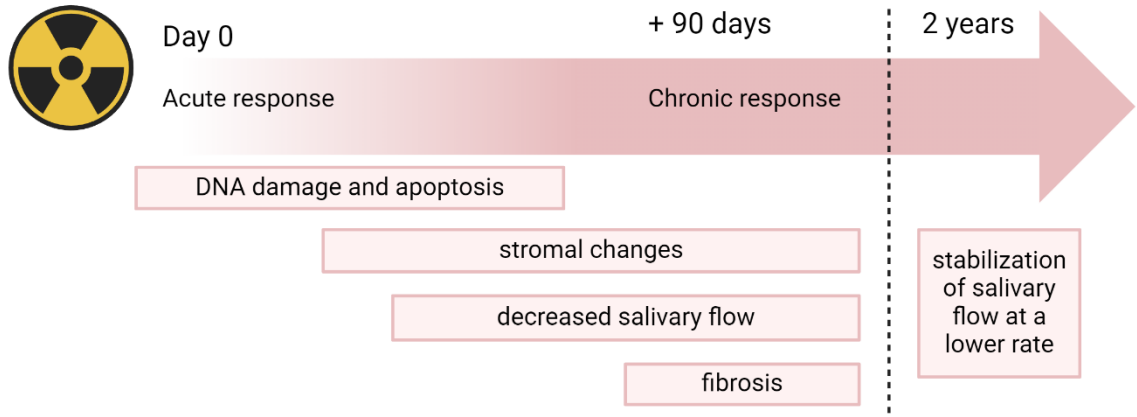
Post-treatment, the patients in Denmark are followed for five years to monitor treatment-failure, recurrence, treatment-related side effects, and rehabilitation in a standardized follow-up program[69]. In Denmark, 23% of the OPSCC patients experience a recurrence, with the majority being locoregional (16%)[70]. Salvage therapy (e.g., TORS following previous RT) in case of recurrence is challenging, with limited possibilities to offer curatively-intended treatment, and the survival following recurrence is very poor regardless of HPV-status[70]. Early detection of recurrences is the key to improving the chances of curative treatment, but despite regular clinical examinations, the ability to detect recurrent disease is limited. Circulating tumor HPV DNA (ctHPV DNA) measurements in plasma is a promising new biomarker as it has demonstrated high sensitivity in detecting recurrence and, importantly, at Rigshospitalet it is possible to

detect the recurrence three to four months earlier than when using the otherwise standardized options[71,72]. Consequently, a prospective, randomized trial has just been initiated at Rigshospitalet to investigate the use of ctHPV DNA as part of the follow-up regimen (Regional Scientific Ethical Committee H-23071576).

Treatment-related toxicities: xerostomia and salivary gland damage

CRT is the traditional treatment regimen for OPSCC in Denmark, and most patients in Denmark and worldwide receive radiation-based treatment[59], which severely impacts QoL and causes long-term side effects[73–75]. The salivary glands are especially radiation-sensitive and are often damaged by radiation, which promotes both acute and chronic responses characterized by inflammation, interstitial fibrosis, glandular shrinkage, loss of acinar cells, salivary gland stem cells and blood vessels leading to salivary gland hypofunction and hyposalivation, and xerostomia or "dry mouth syndrome"[76–78]. See **Figure 6**.

Figure 6. **Radiation damage to the salivary glands over time.** Radiation causes both acute and chronic damage to the salivary glands. Chronic changes include fibrosis, glandular atrophy, and persistent hyposalivation and xerostomia. These changes significantly impact the overall oral health and QoL.



Abbreviations: DNA, deoxyribonucleic acid, QoL, quality of life.

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**Figure inspired by Jasmer et al., *J Clin Med*, 2020[78].

Even though intensity-modulated radiotherapy (IMRT) can reduce irradiation to the parotic glands[79], xerostomia is the most frequent side effect following RT and it has been estimated to affect more than 80% of patients[77]. The risk of salivary gland damage is associated with the level of delivered RT and is influenced by several treatment and patient-related factors such as the target tissue/anatomical area in question, the total radiation dose, and dose per fraction[80,81]. A cumulative dose of 24-26 Gy to the parotic glands and 35 Gy to the submandibular glands have been recommended to allow the salivary glands to recover and thereby diminish the side effects[81,82].

Salivary gland function and salivation are crucial for overall oral health: they aid in food digestion and swallowing, protects the teeth and oral mucosa, and moistens the palate for articulation[83]. Radiation-induced salivary gland hypofunction is characterized by reduced saliva production and impaired saliva composition[84,85] and has a significant impact on the daily functioning and overall QoL among cancer survivors[73]. Salivary gland hypofunction and hyposalivation increase the risk of oral infections and dental decay, impair chewing and swallowing with risk of malnutrition, and affect speech and sleep quality[64,76,85]. Consequently, many patients are restricted in their daily activities and social life[86]. Currently, treatment options for patients with salivary gland hypofunction are limited to symptomatic management. Consequently, there is a significant and unmet need for novel, disease-modifying therapeutic approaches for hyposalivation and xerostomi[87].

Prognostication and treatment de-escalation strategies

Standard treatment regimens are associated with a high burden of side effects and reduced QoL[73], and since most patients with HPV+ OPSCC have an excellent prognosis, there has been a growing interest in personalized and de-escalated treatment strategies to reduce the treatment-related toxicities without compromising the prognosis for selected patients in clinical trials[88,89]. However, this is complicated by the fact that, despite a high survival for HPV+ OPSCC in general, a subset of patients (15%) still has a worse prognosis with both locoregional recurrences and distant metastases[70].

De-escalation strategies in clinical trials vary but encompass largely dose-reducing or replacing CRT, TORS, and induction chemotherapy. To reduce toxicity, substitution of Cisplatin with Cetuximab has been investigated as a de-escalation strategy in low-risk HPV+ OPSCC but has reduced tumor control[90,91]. RT deintensification strategies to reduce RT-related toxicities include, among others, parotid sparring RT[79]. The use of TORS as a de-escalation strategy to preserve swallowing function is of debate, as the ORATOR Trial did not demonstrate its superiority based on MD Anderson Dysphagia Inventory (MDADI) scores[66].

However, as previously noted, the TORS group underwent neck dissection at the time of surgery or within two weeks, and as a consequence, patients with adverse prognostic features received multimodality treatment. However, the results of the ongoing QoLATI study are highly anticipated as swallowing function is assessed by both MBS and FEES.

No international consensus on how to stratify HPV+ OPSCC patients in clinical trials exists, and even the definition of HPV-status differs between de-escalation studies[88,89]. Since p16+/HPV+ patients have the best prognosis, using p16 as a stand-alone in stratification might incorrectly classify 10% of p16+ patients as HPV+ [12,92]. Other risk factors that could be considered are smoking[93] and oropharyngeal subsite[51]. Precise risk stratification is crucial if de-escalation strategies are to succeed and is important in a broader perspective if these strategies are to be implemented in diverse geographical areas with differences in demographics such as smoking prevalence and differences in p16/HPV discordance[12].

Screening and vaccine

A way to achieve widespread prevention of HPV-driven cancer, including HPV+ OPSCC, is through national vaccination programs. The quadrivalent HPV vaccination Gardasil®, covering the genotypes HPV16, -18, -6 and HPV11 was implemented in the Danish Children Vaccination Programme in 2009 to girls to prevent HPV-driven cervical cancer[23]. From 2017 it was replaced with the ninevalent vaccine covering the additional genotypes HPV31, -33, -45, -52, and HPV58[94] which covers more than 95% of HPV+ OPSCCs[16,27] and in 2019 boys were included[23]. Including boys in the vaccination programs is important to mitigate the risk of HPV+ cancer, since this approach will not only protect the vaccinated boys themselves but aid vaccine coverage to reach herd immunity levels as well[95].

Due to the natural history of OPSCC spanning over 30-40 years, the effect of HPV vaccination on the risk of HPV+ OPSCC is still immature, but studies indicate that the vaccine reduces the presence of oral HPV[96,97]. This is encouraging and supports the belief that HPV vaccination is efficient in preventing HPV+ OPSCC as well, but it will most likely not be evident in the incidence rates for the next decades[98]. This underlines that the disease burden of HPV+ OPSCC will continue within the coming years at the cost of both the patients and society. It is immensely crucial to continue expanding the knowledge on the burden of HPV+ OPSCC among the public, healthcare professionals, and authorities to ensure continuous awareness, medical recognition of HPV+ OPCC, and continued political attention[99–102]. Still, only girls are offered catch-up vaccination, while this is not available for boys[103]. In addition, the incidence might be affected by vaccination pressure which may cause the virus to adapt to new vaccine-resistant strains[104].

Another method to prevent HPV-driven cancer is through screening. The purpose of cancer screening is to identify patients with either precancerous lesions or early-stage disease, allowing single-modality and more amenable treatment. On the other hand, late diagnosis of head and neck cancer and OPSCC are associated with higher tumor volume and more advanced stage at diagnosis[105,106], which is related to poorer survival and increases the necessity of multimodality treatment with increased morbidity[42]. Detecting HPV+ OPSCC as early as possible could lead to diagnosing the cancer at earlier stages with lower tumor burden, potentially improving the prognosis and reducing treatment side effects.

Cervical cytology screening has been very successful in reducing the cervical cancer incidence rates[107,108]. However, several differences exist between cervical cancer and OPSCC driven by HPV. The development of HPV+ OPSCC is associated with oral infection with HPV, but the natural history from transient oral HPV infection to persistent infection and the development of OPSCC is poorly understood compared to cervical HPV infection and cancer[21]. Understanding this multi-step progression is further complicated by the absence of identifiable pre-cancerous lesions, and the fact that OPSCC development typically spans 30 to 40 years from initial exposure[21]. In addition, oral HPV infection tends to be more transient than cervical-genital HPV infection and are often cleared within 1-2 years[109], yet studies have shown that persistent infection as indicated by the presence of oral HPV antibodies or DNA is associated with OPSCC[110,111].

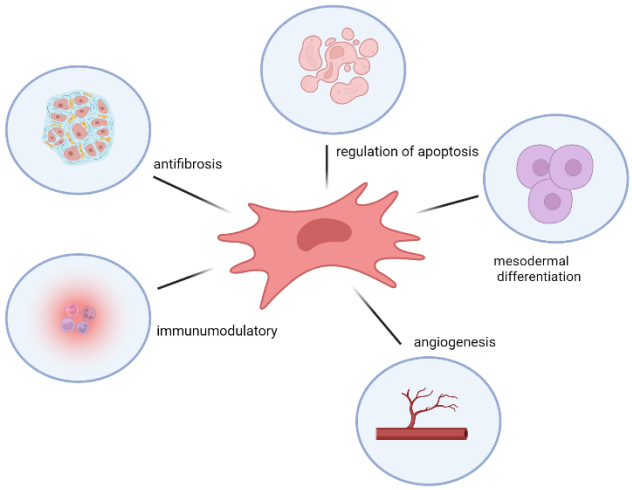
These circumstances complicate the possibilities for screening for HPV+ OPSCC. As with cervical screening, cytological screening has been investigated, but even in selected high-risk populations, cytological testing was not able to reliably screen for OPSCC[112]. Another strategy is detection of oral HPV infection, but this has shown both low sensitivity and specificity, suggesting it would imply both many false negatives and false positives[109,113]. The presence of sera positive HPV16 E6 antibodies is associated with the development of later HPV+ OPSCC, but they can be detected decades before the development of cancer and the far majority will not develop OPSCC[114,115]. In conclusion, appropriate screening on a population level remains challenging, but might be more feasible in high-risk populations.

Mesenchymal stem cells and radiation-induced xerostomia

Mesenchymal stem cells (MSCs) are fibroblast-like, multipotent, adult stem cells, and were first described in the bone marrow, bone marrow-derived MSCs (BSCs), over 40 years ago by Friedenstein et al., but can reside in most connective tissue, including adipose tissue, and adipose-derived mesenchymal stem cells (ASCs)[116,117]. ASCs have several advantages compared to BSCs: they are easily harvested by a simple liposuction in local anesthesia, contains a high concentration of MSCs, and they grow faster during ex vivo

expansion[118]. The definition of MSCs is their ability to adhere to plastic surfaces, presence (CD73, CD90, CD105) and absence (CD14, CD34, CD45) of specific surface molecules, and potential to differentiate into various cells, such as osteoblasts, chondrocytes, and adipocytes, from the mesodermal germal layer, highlighting their potential for regenerative medicine[119,120]. Rather than playing a crucial role in engraftment[121], studies have demonstrated that MSCs exhibit various microenvironmental reorganizational properties through both paracrine and trophic mechanisms; see **Figure 7**. These properties include anti-inflammatory, regulatory apoptotic, immunomodulatory, and angiogenetic effects, although the exact mechanisms of action are not yet fully understood[117,122].

Figure 7. Mechanisms of MSCs. MSCs exert their therapeutic effect primarily through paracrine signaling and differentiation (engraftment). MCSs can give rise to mesodermal cells. Through paracrine signaling, MSCs can reduce fibrosis, promote angiogenesis, regulate apoptosis, and modulate the immune system. As a result, transplanted MSCs induce tissue regeneration and repair.



Abbreviations: MSCs, mesenchymal stem cells.

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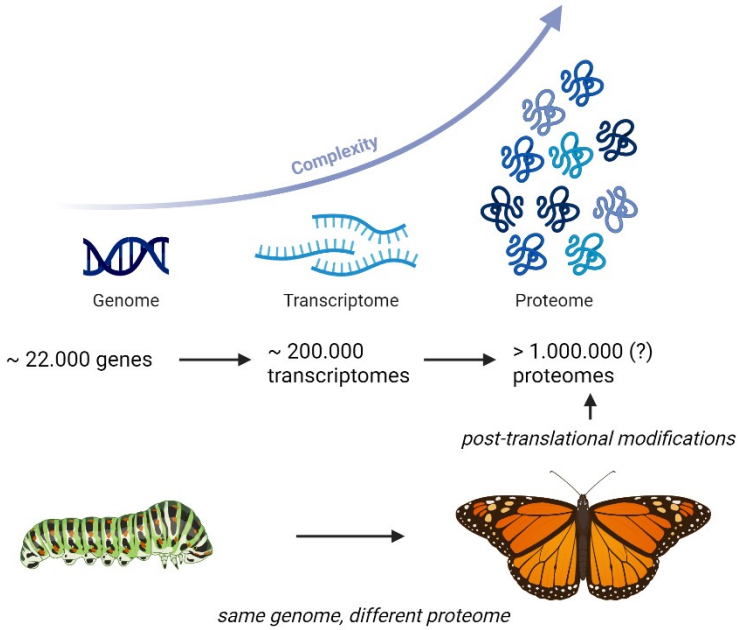
Due to their regenerative potential, MSCs have been investigated in numerous clinical trials for various disorders, such as wound healing, Crohn's disease, and fistulas, with promising results and only minimal side effects[123–125]. MSCs are believed to be immune evasive since they express low levels of major histocompatibility complex (MHC) class I and lack expression of MHC class II[126,127]. The use of allogeneic, immune-evasive MSCs allows a standardized, ready-to-use product, i.e., "off-the-shelf" treatment.

Our research group has completed several encouraging and original studies investigating the safety and efficacy of intraglandular ASCs treatment for radiation-induced salivary gland hypofunction after head and neck cancer to regenerate the salivary gland function and enhance the saliva production[128–130]. Our initial pilot study was a randomized, placebo-controlled trial with 30 patients, which assessed the safety and efficacy of autologous ASCs[130]. The study showed that intraglandular treatment with ASCs was safe without serious adverse events (SAEs) and was associated with an increase in the saliva flow rate (SFR) of 33-50%[130]. This was reflected in the patient-reported outcome measurements (PROMs), where patients who received ASCs experienced diminished trouble in eating both at short-term follow-up after four months and after long-term follow-up[130,131]. But, we experienced considerable individual variability in the manufacturing of autologous ASCs, particularly in terms of cell yield, cell quality, and expansion time. To address these challenges and to avoid the need for each patient to undergo liposuction, we shifted to allogeneic ASCs. The safety of allogenic ASCs from healthy donors as a standardized treatment was investigated in a *first-in-man* feasibility study[129]. This study proved allogenic ASCs to be safe without SAEs and with promising efficacy results with 46% increase in the saliva production and improved PROMs regarding dry mouth, sticky saliva, and swallowing[129]. Moving forward, a single-center, blinded, randomized, placebo-controlled, phase II study to investigate the efficacy of treatment with ASCs for radiation-induced salivary gland hypofunction and xerostomia in previously irradiated head and neck cancer patients was initiated[128,132]. The study included 120 patients who were randomized 1:1 to receive either ASCs or placebo in the submandibular glands. ASCs therapy significantly increased the saliva production by 38%, although the treatment was not superior to placebo after four months[128]. Intraglandular BSC therapy has shown an encouraging effect on radiation-induced xerostomia as well[133]. However, the long-term effect of intraglandular MSC therapy has not been investigated in larger, randomized, clinical trials.

The salivary proteome and mesenchymal stem cells

Proteomics are the large-scale study of proteins, including both their structure and functions[134]. It encompasses the identification, quantification, and analysis of the entire protein complement in a unit: for example, in a cell, tissue, or organism[134]. While genomics is relatively static, proteomics provides extensive and complex overview of the dynamic, post-translational protein landscape within a biological context; see **Figure 8**. Thus, researchers can gain insights into how proteins work together or how they change in response to various conditions or diseases. In contrast, single protein biomarkers (e.g., C-reactive protein) serve as specific indicators and provide more targeted information.

Figure 8. **Proteomics and the relation to genomics**. Genomics is the study of the entire DNA sequence that is transcribed into RNA. Transcriptomics measure the gene expression at the RNA level. Proteomics capture the complexity of protein expression, including post-translational modifications, offering a more functional insight.



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Proteomics can be investigated in different ways providing distinct information: measuring the abundance of different proteins, comparing proteomes between different samples or cohorts (conditions), structural analysis, changes in proteins' expression under a given condition, protein interaction, and functional analysis. The most used method to analyze proteomics is mass spectrometry. Briefly, this technique includes ionization and enzymatic digestion of proteins into smaller peptides, which are then separated by their mass-to-charge ratio followed by protein identification[135].

Saliva contains a diverse array of proteins reflective of the overall oral health[136]. As a result, investigating the saliva proteome holds promises for e.g., early detection, monitoring disease progression, and tailoring personalized treatment strategies. The whole salivary proteome encompasses secreted proteins from all the salivary glands (parotid, submandibular, sublingual, and minor salivary glands) and therefore does not reflect proteins' alterations in specific salivary gland tissue. Studies have shown that radiation alters specific proteins in the saliva[137–140]. These changes include alterations in the levels of various proteins such as amylase and mucins, as well as inflammatory markers associated with periodontal status and the feeling of oral dryness[137–140].

Treatment with ASCs may induce significant changes in the saliva proteome as a possible mechanism of action, even though the proteome after treatment with ASCs is not restored to normal[138]. The changes observed following ASC include upregulated proteins involved in tissue regeneration, immune system, and cell growth[138]. These findings have not yet been validated in larger, clinical randomized trials.

Aims and hypotheses

Paper I: Clinical and Prognostic Differences in Oropharyngeal Squamous Cell Carcinoma in USA and Denmark, Two HPV High-Prevalence Areas

The aim of this study is to investigate if differences exist among OPSCC patients between two high HPV-prevalence areas but with distinct populations and healthcare systems – in terms of demographics, clinical characteristics, treatment modalities, and prognosis at the UTMDACC and in Eastern Denmark. In Denmark, patients with OPSCC are diagnosed and treated within a universal healthcare system with a focused budget, representing an unselected and broad population. In contrast, the UTMDACC operates within an insurance-based system, serving a highly selected, resourceful patient population. Additionally, UTMDACC is the top-ranked cancer center in the U.S.[141], with a more flexible budget, attracting patients from across the country. Differences in treatment guidelines exist; for example, neoadjuvant chemotherapy is available at UTMDACC but is not currently an option in Denmark.

The hypotheses are:

1) Differences in demographics, clinical characteristics, and treatment modalities exist between the U.S. and Eastern Denmark, despite both being high HPV-prevalence areas, associated with the disparities in the healthcare systems and treatment practice patterns.
2) The prognosis for OPSCC patients varies between the U.S and Eastern Denmark, despite both regions having a high HPV prevalence.

Paper II: Mesenchymal Stromal/Stem Cell Therapy Improves Salivary Flow Rate in Radiation-Induced Salivary Gland Hypofunction in Preclinical In Vivo Models: A Systematic Review and Meta-Analysis

The aim of this study is to review the current literature on the safety and effectiveness of MSCs as a therapy for radiation-induced salivary gland damage and hypofunction in animal models.

The hypotheses are:

1) MSC therapy for radiation-induced salivary gland damage in animal models is safe.
2) MSC therapy can restore the salivary gland function following radiation-damage in animal models.

Paper III: Long-term Effectiveness and Safety of Mesenchymal Stromal Cell Therapy for Radiation-induced Hyposalivation in Head and Neck Cancer Survivors: A Randomized, Placebo-controlled, Phase-2 Trial

The aim of this study is to investigate the long-term effectiveness and safety of intraglandular treatment with allogeneic ASCs in the submandibular glands to restore the salivary gland function in patients with radiation-induced salivary gland damage and hypofunction in previously irradiated head and neck cancer patient in a randomized, placebo-controlled trial. The study represents the long-term results of the previously published, primary four-month endpoint (MESRIX-III)[128].

The hypotheses are:

1) Intraglandular allogeneic ASC therapy can effectively restore the salivary gland function following radiation-induced salivary gland damage in head and neck cancer patients compared to placebo.
2) Intraglandular allogeneic ASC therapy to restore the salivary gland function following radiation-induced salivary gland damage in head and neck cancer patients is safe.

Paper IV: No Changes in the Salivary Proteome Composition Detected After Intraglandular Mesenchymal Stem Cell Therapy for Radiation-Induced Xerostomia in Previous Head and Neck Cancer Patients: A Randomized Phase 2 Trial

The aim of this study is to investigate the mode of action of intraglandular treatment with allogeneic ASCs in the submandibular glands to restore the salivary gland function in patients with radiation-induced salivary gland damage and hypofunction in previously irradiated head and neck cancer patients in a randomized, placebo-controlled trial evaluated by changes in the salivary proteome.

The hypotheses are:

1) Intraglandular ASC therapy induces changes in the composition of the salivary proteome in head and neck cancer patients with radiation-induced salivary gland damage and hypofunction compared to placebo.
2) The composition of the salivary proteome is altered in previously irradiated head and neck cancer patients compared to healthy controls.

Materials and Methods

Paper I: Clinical and Prognostic Differences in Oropharyngeal Squamous Cell Carcinoma in USA and Denmark, Two HPV High-Prevalence Areas

Study design and population

The study was a retrospective, cohort study including two distinct cohorts of patients with OPSCC from 2015-2020 in two high-HPV-prevalence areas: one population-based cohort from Eastern Denmark, and one selected cohort from the University of Texas MD Anderson Cancer Center, USA (UTMDACC).
The Copenhagen cohort was approved by The Regional Scientific Ethical Committee (H-20072877). The Houston cohort was approved by the Institutional Review Board of the UTMDACC (protocol PA 14-0947).

Outcomes

The primary endpoints of the study were to compare the demographic and clinical characteristics and treatment modalities given between the two centers. The secondary endpoints were to compare the prognosis evaluated as the three-year OS and three-year recurrence-free interval (RFI) and subgroup analyses of low-risk OPSCC patients (T1-T2/N0/M0) and high-risk patients (stage III-IV).

Statistics

Statistical analyses were performed in R Studio (version 4.1.3). Associations between variables were evaluated with either Pearson's chi-squared, Fischer's exact test, or *t*-test depending on the type of data and sample size. A p-value < 0.05 was considered statistically significant. OS and RFI were evaluated with Kaplan-Meier curves, the log-rank method, and by uni- and multivariable Cox regression analyses.

A comprehensive description of the material and methods can be found in Paper I.

Paper II: Mesenchymal Stromal/Stem Cell Therapy Improves Salivary Flow Rate in Radiation-Induced Salivary Gland Hypofunction in Preclinical In Vivo Models: A Systematic Review and Meta-Analysis

Study design and outcomes

This study was a systematic review and meta-analysis including preclinical in vivo models that assessed the effect of MSC therapy to restore the salivary gland function following radiation-induced salivary gland

damage. The primary endpoint was effect on SFR while secondary endpoints were effect on salivary gland morphology and histology, saliva composition, paracrine effects, and safety evaluated as adverse events. The study protocol was published and registered at PROSPERO (www.crd.ac.uk/prospero, CRD42021227336) prior initiation of this study [142].

Systematic search and data extraction

The search string was created using medical subject headings (MeSH), Emtrees, and text words connected to MSCs, radiation-induced salivary gland hypofunction, damage or dysfunction, and xerostomia.

Two authors (CH and ALFC) independently searched PubMed and Embase and screened studies for eligibility. Details of the preclinical in vivo model (species, sex, sample size, age), the study design (controlled, uncontrolled, randomized, blinding), irradiation details (dose, days from irradiation to MSC therapy), MSC therapy (type, concentration, administration route, follow-up time), statistical analysis, and outcomes (salivary flow rate, molecular) were extracted from each study.

Quality assessment and risk of bias

Quality assessment was performed using the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines[143], while risk of bias was assessed using the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) guidelines[144].

Data analysis

The random effect meta-analysis adjusted to Hedge's g was used to evaluate the efficacy of MSC therapy on salivary flow rate. A standardized mean difference (SMD) was used to assess the effect on SFR. We investigated the following subgroups: species, strain, sex, administration route, age, radiation duration, frequency of treatment, radiation dose, and time from radiation to MSC therapy.

A comprehensive description of the material and methods can be found in Paper II.

Paper III: Long-Term Effectiveness and Safety of Mesenchymal Stromal Cell Therapy for Radiation-Induced Hyposalivation in Head and Neck Cancer Survivors: A Randomized, Placebo-Controlled, Phase-2 Trial

Study design

The study was the long-term follow-up of the investigator-initiated, single-center, randomized, placebo-controlled trial investigating the efficacy and safety of allogeneic intraglandular ASCs for radiation-induced salivary gland damage and hypofunction[128]. The study was initiated in January 2020 and conducted collaboratively by my fellow PhD student, Kathrine K. Jakobsen, and myself. The primary endpoint was the effect after four months and has been previously published [128]. The study was approved by the National Ethics Committee (Protocol number: 1802872), The Danish Medical Agency (EudraCT: 2018-000348-24), and the Danish Data Protection Agency (Protocol number P-2020-1164). The trial was monitoring by an external Good Clinical Practice unit (GCP). The study protocol has been published[132].

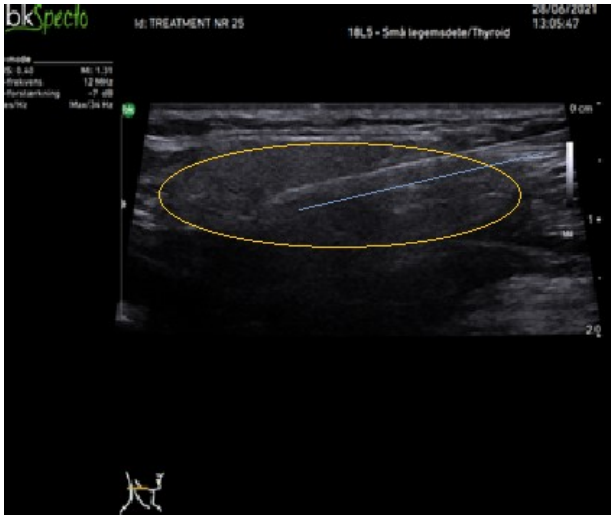
Study population

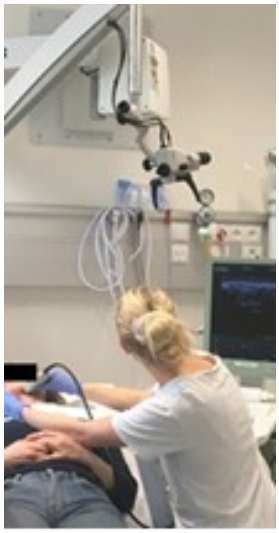
The study included 120 previously irradiated, head and neck cancer patients with radiation-induced salivary gland damage and hypofunction[128]. The inclusion criteria were clinically reduced salivary flow rate, a minimum of two years since radiation therapy, age from 18-75 years, without recurrence, and informed consent. Patients were excluded if they had any cancer in the previous four years (excluding the head and neck cancer), received xerogenic medicine, had penicillin or streptomycin allergy, had prior salivary gland disease or surgery, were pregnant or breastfeeding, were currently smoking, or were abusing alcohol[128].

Interventions and study flow

Patients were randomized 1:1 to receive either placebo (CryoStor10, BiolifeSolutions, containing 10% Dimethyl sulfoxide [DSMO]) or 25 million allogeneic ASCs in each submandibular gland[128]. The interventions were performed freehand guided by ultrasound, and without anesthesia; see **Figure 9**.

Figure 9. **Intervention** (with permission from the patient). The intervention was performed as an out-patient visit and done freehand guided by ultrasound, and without anesthesia. The yellow circle shows the submandibular gland while the blue line shows the needle during the injection of ASCs.

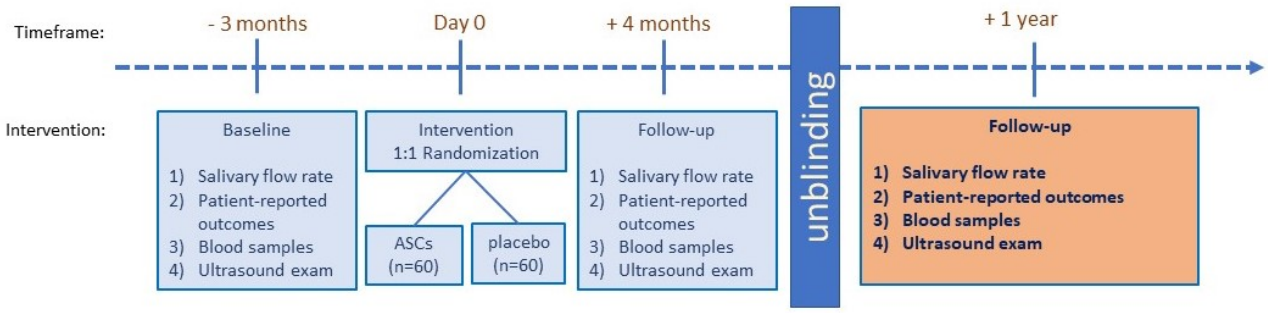




Abbreviations: ASCs, adipose-derived mesenchymal stem cells.

At baseline, the unstimulated whole salivary flow rate (UWS) and the stimulated whole salivary flow rate (SWS) were evaluated by sialometry. PROMs were evaluated with the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire - Head and Neck 35 (EORTC QLQ-H&N35), and xerostomia questionnaire (XQ). An ultrasound of their submandibular glands was made, and presence of human leukocyte antigen (HLA) antibodies evaluated in a blood sample. Effect of ASC therapy at four months was the study's primary endpoint and is previously published[128]. At four months, both patients and study personnel were unblinded to the treatment, and patients were subsequently followed for a total of 12 months post-intervention. The study flow is illustrated in **Figure 10**.

Figure 10. **Study flow.** The study was initiated in January 2020 and conducted collaboratively by my fellow PhD student, Kathrine K. Jakobsen, and myself. The primary endpoint was the effect after four months and has been previously published (shown in blue)[128]. After four months, the study became unblinded and the long-term effect was evaluated 12 months after intervention (shown in orange).



Abbreviations: ASCs, adipose-derived mesenchymal stem cells.

Outcomes

The primary endpoint of this study was the long-term effectiveness of intraglandular ASC therapy evaluated as the change in mL/min in UWS. Secondary endpoints were the long-term change in SWS and change in the PROMs XQ and EORTC QLQ-H&N35, domains HNSS (sticky saliva), HNDR (dry mouth), and HNSW (swallowing). Safety was evaluated as the development of de novo donor-specific HLA antibodies (DSA) and treatment-related SAEs.

Statistics

A statistical analysis plan was made prior to data analysis. Data was analyzed with a multilevel repeated measurements mixed effects model, with the participants as a random effect factor and the outcome variable as a dependent variable. Based on a restricted maximum likelihood model, the time was set as a fixed factor (month 0, 4, and 12). Considered significant were p-values > 0.05. A sensitivity analysis was performed for missing values using a simplistic non-responder technique with baseline values carried forward.

Paper IV: No Changes in the Salivary Proteome Composition Detected After Intraglandular Mesenchymal Stem Cell Therapy for Radiation-Induced Xerostomia in Previous Head and Neck Cancer Patients: A Randomized, Phase 2 Trial

Study design

The study was an add-on to the randomized, placebo-controlled trial investigating the efficacy and safety of allogeneic intraglandular ASCs for radiation-induced salivary gland damage and hypofunction[128]. The study was approved by the National Ethics Committee (Protocol number: 1802872), The Danish Medical Agency (EudraCT: 2018-000348-24), and the Danish Data Protection Agency (Protocol number P-2020-1164). The trial was monitoring by an external Good Clinical Practice unit (GCP). A study protocol to this additional study has been submitted[145].

Study population

As described previously, the study included 120 previously irradiated head and neck cancer patients with radiation-induced hyposalivation[128]. Salivary proteomic output from 10 healthy controls was obtained from our previous study[138].

Interventions and study flow

As descried previously, patients were randomized 1:1 to receive either placebo (CryoStor10, BiolifeSolutions, containing 10% DMSO) or 25 million allogeneic ASCs in each submandibular gland [128]. At baseline and four months after intervention, UWS samples were collected and immediately put on dry ice and stored at -80^{o}C.

Outcomes

The primary endpoint of this study was change in the salivary proteome composition following ASC therapy compared to placebo four months following ASC therapy. Secondary endpoints included 1) change in the salivary proteome composition following ASC therapy at baseline compared to four months, 2) evaluation of the salivary proteome composition in sub-groups associated with enhanced clinical effect (smoking, mean radiation dose to the four large salivary glands, development of donor specific antibodies and clinical

effect of 30% or more), and 3) descriptive analysis of the salivary proteome composition in irradiated patients compared to healthy controls.

Mass spectrometry analysis and protein identification

The saliva samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Significant differentially expressed proteins were reviewed in the Uniprot database. The salivary proteome composition in the group receiving ASC therapy was compared to the salivary proteome composition in the group receiving placebo group at four months after intervention. Results were visualized with PCA plots and volcano plots, and significance levels between the groups were evaluated with double-sided t-test. Functional annotation was evaluated in the Database of Annotation, Visualization, and Integrated Discovery (DAVID) encompassing Gene Ontology Biological Process (GO:BP) using cluster analysis.

Subgroup analyses of ASC-treated patients were chosen based on the clinical effect on increase in UWS reported at four months, with effect on UWS being associated with smoking, mean radiation dose, and development of DSAs[128]. We hypothesized that a change in the salivary proteom composition would be more evident in patients with a substantial clinical effect. Hence, subgroup analysis within the ASC group included never smokers vs. ever smokers, patients who did not develop DSAs vs. patients who did not, patients who received a mean radiation dose <40 Gy vs. >40 Gy and those with a substantial clinical effect on UWS of 30% or more vs. clinical effect on UWS <30% at four months. Lastly, a comparison of the irradiated salivary proteome at baseline compared to the healthy salivary proteome composition was made, with an emphasis on Cystatin-D, -S, -SN and -SA, Glutaredoxin-1, Histatin-1, Lipocalin-1, and statherin which were significantly upregulated in healthy controls compared to irradiated patients in our previous study[138].

Summary of the key results

Paper I: Clinical and Prognostic Differences in Oropharyngeal Squamous Cell Carcinoma in USA and Denmark, Two HPV High-Prevalence Areas

The study included 2,484 patients: a large cohort of 1,216 patients with OPSCC from Eastern Denmark and a corresponding large cohort of 1,268 patients with OPSCC from UTMDACC. The Copenhagen cohort demonstrated:

- lower HPV prevalence (63% vs. 88%)
- more females (26% vs. 11%)
- older age (median age 62 years vs. 60 years).
- more ever smokers (76% vs. 47%).
- more tumors located in other OPSCC subsites than palatine tonsil and base of tongue (18% vs. 4%)
- more tumors at higher T-stages (T3-T4: 34% vs. 25%).
- more tumors with advanced nodal disease (N2-N3: 26% vs. 21%).
- more tumors at higher overall stage (III-IV: 29% vs. 16%).
- more single modality therapy (RT alone 30% vs. 15%; surgery alone 16% vs. 9%).
- worse three-year OS (85% vs 95%, log-rank $p<0.001$), but not in the multivariable analysis, (hazard ratio [HR] 1.21, $p=0.23$).
- worse three-year recurrence-free interval (83% vs. 91%, log-rank $p<0.001$), and in the multivariable analysis (HR of 1.74, $p=0.003$)

We found significant differences in clinical characteristics and treatment modalities given in subgroup analyses of low-risk (T1/T2N0M0) and high-risk (stage III-IV) patients. Significantly worse OS and RFI were found for high-risk patients, but not for low-risk patients. In low-risk patients, significantly more patients in the Copenhagen cohort received single-modality treatment.

Main findings

Despite both cohorts having a high HPV prevalence, they were significantly different regarding their demographic, clinical characteristics, and treatment modalities given, which was reflected in significant differences in the three-year prognosis.

Paper II: Mesenchymal Stromal/Stem Cell Therapy Improves Salivary Flow Rate in Radiation-Induced Salivary Gland Hypofunction in Preclinical In Vivo Models: A Systematic Review and Meta-Analysis

The systematic review included 16 studies of which 13 were included in the meta-analysis. Four studies used BSCs, 10 studies used ASCs, and two studies used salivary gland tissue-derived MSCs. Treatment with MSCs was associated with:

- no SAEs.
- increased SFR with an overall effect of 6.99 SMD, (95% CI: 2.55 to 11.42).
- improvements in acinar tissue, vascular areas, and paracrine factors.
- Reduction in apoptotic cells.
- Intraglandular injection were superior to intravenous injection.

Main findings

Treatment with MSCs in preclinical in vivo models was safe and significantly improved the salivary gland function and SFR. Additionally, MCSs induced structural and potentially regenerative changes in salivary gland tissue following radiotherapy.

Paper III: Long-term Effectiveness and Safety of Mesenchymal Stromal Cell Therapy for Radiation-Induced Hyposalivation in Head and Neck Cancer Survivors: A Randomized, Placebo-controlled, Phase-2 Trial

The baseline characteristics of the enrolled patients are previously published[128]. Briefly, a total of 120 patients were included; most were males (73%) with a median duration since RT of 4.3 years (IQR 2.8 to 6.8) and had an HPV+/p16+ OPSCC (78%)[128]. Of these, 117/120 (97.5%) were assessed at 12 months after intervention. The long-term effect of treatment with ASCs was associated with:

- no difference in change in UWS compared to placebo (0.007 mL/min; 95% CI [-0.02 to 0.0]3).
- a significant and clinically relevant reduction in the symptom burden for dry mouth compared to placebo (difference in change was -5.93 units; 95% CI [-10.62 to -1.22]).
- no differences in change were observed in the PROMs regarding sticky saliva, swallowing, or xerostomia compared to placebo.
- no increased risk of treatment-related SAEs compared to placebo.
- Transient immune response for those who developed donor specific antibodies.

Main findings

Treatment with intraglandular ASCs alleviated the subjective feeling of dry mouth compared to placebo, but we did not find that ASCs were superior to placebo to restore salivary gland function as evaluated by objective measurements. Treatment with ASCs was safe, without long-term SAEs.

Paper IV: No Changes in the Salivary Proteome Composition Detected After Intraglandular Mesenchymal Stem Cell Therapy for Radiation-Induced Xerostomia in Previous Head and Neck Cancer Patients: A Randomized Phase 2 Trial

All 120 enrolled patients were assessed at four months following intervention[128]. Of these, 89 (74%) were included in the proteomic analyses while 30 were excluded due to inconsistent sample preparation and one patient did not produce any saliva at four months. Treatment with ASCs was associated with the following.

- No differentially expressed proteins were observed in the group receiving ASC therapy compared to placebo at four months.
- Several proteins (Cystatin-S, Cystatin-D, Cystatin-SA, Cystatin-SN, Glutaredoxin-1, and Lipocalin-1) that are upregulated in the healthy salivary proteome compared to irradiated patients were insignificantly upregulated in the ASC group compared to placebo at four months, although the levels did not reach that observed in healthy saliva.
- No changes in the salivary proteome were observed following ASC therapy at four months compared to baseline.
- Changes in the salivary proteome in the ASC group were not associated with smoking, mean radiation dose to the four large salivary glands, or clinical effect (>30% increase UWS).
- An insignificant enrichment of protein also upregulated in healthy saliva was detected in ASC-treated who did not develop DSAs compared to those who did experience an immunological response with development of DSAs.
- An increased metabolic rate and oxidative stress were observed in irradiated patients compared to healthy controls.
- In irradiated patients, important salivary proteins (cystatins and lipocalin) were upregulated in patients who had received a mean radiation dose to the four salivary glands below 40 Gy compared to more than 40 Gy, but the salivary proteome was not affected by smoking status.

Main findings

The study did not demonstrate changes in the salivary proteome composition following treatment with intraglandular ASCs compared to placebo, but treatment with ASCs did tend to induce upregulation of important salivary proteins upregulated in healthy controls. No association of smoking, mean radiation dose, or clinical effect was established on the mode of action of ASCs, but immunological tolerance tended to be associated with upregulation of salivary proteins upregulated in healthy saliva. Important functional differences were observed between irradiated patients and healthy controls, and patients who had received a dose below 40 Gy had a significant upregulation of proteins related to the oral immune system and taste perception.

Discussion

Paper I: Clinical and Prognostic Differences in Oropharyngeal Squamous Cell Carcinoma in USA and Denmark, Two HPV High-Prevalence Areas

Paper I was the largest, retrospective, cohort study investigating if clinical, treatment, and prognostic differences exist between two high HPV-prevalence OPSCC cohorts: one cohort from the highest-ranking cancer center in in the U.S[141], UTMDACC, and one cohort from Eastern Denmark, representing not only distinct population, but distinct healthcare systems. The diverse healthcare systems might introduce a selection bias, with an unselected patient population in Eastern Denmark and a highly-selected patient population in the UTMDACC cohort. Studies have shown that insurance status is predictive of tumor stage and overall survival, and that patients without private insurance present at higher tumor stages, select treatment sites with poorer survival outcomes, and have a worse overall survival[146,147].

Given the current emphasis on optimizing treatment stratification for either de-escalation or escalation in clinical trials[88,89], accurate patient stratification is essential. If results from clinical studies are to be broadly applicable across different OPSCC cohorts, it is crucial to understand the extent to which these cohorts are comparable. Our results showed significant differences in demographic and clinical characteristics despite both cohorts having a high HPV-prevalence, both in the overall OPCC cohorts and in low- and high-risk patients potentially eligible for de-escalated or escalated treatment, respectively. This is in line with other studies[148], and it might be important to include multiple factors for stratification to ensure reproducibility across diverse geographical areas.

The study revealed significant differences in treatment modalities given, with single-modality RT or TORS being more prevalent in the Copenhagen cohort. This may indicate differences that influence the treatment selection not included in this study, such as comorbidities. Differences in practice patterns might play a role and have recently been reported between a Danish and a Toronto cohort[148]. It is noteworthy that significantly more patients with low-risk OPSCC (T1-T2/N0/M0) received single-modality treatment in the Copenhagen cohort, yet no increased HR was found for both overall survival and recurrence-free interval.

The study revealed significant differences in prognosis. Although overall survival was comparable after adjusting for the demographic and clinical differences between the cohorts, the risk of recurrence remained higher in the Copenhagen cohort. This disparity may be multifaceted, potentially influenced by variations in follow-up regimens, differences in the definition and registration of recurrence, and possibly distinct biological factors, although the latter remains speculative. In the Danish universal healthcare system, where each patient is assigned a unique personal identification number, tracking and documenting disease recurrence is feasible even across different treatment sites.

The strengths of the study are that it is the largest cohort study comparing European and American high HPV-prevalence OPSCC populations from diverse healthcare systems. The study has limitations. Importantly, we were not able to use double p16/HPV-positivity in the UTMDACC cohort, which has proven to be superior in prognostication[12]. Utilizing both p16 and HPV-status is recommended, especially in areas with high discordance [12,149]. Second, we did not include socioeconomic status, performance score, or comorbidities, which are prognostic and potentially impact treatment selection. It would be interesting to investigate these factors in future studies, since we believe these vary between the two cohorts, in part driven by the differences in healthcare systems. A slightly longer follow-up time was observed in the Copenhagen cohort (2.3 versus 2.1 years, $p<0.001$), which might impact the prognostic analysis. Although interesting differences were indicated in both low- and high-risk OPSCC patients, the sample size of especially the low-risk group was too small with too few events to make strong conclusions but were rather hypothesis generating.

Paper II: Mesenchymal Stromal/Stem Cell Therapy Improves Salivary Flow Rate in Radiation-Induced Salivary Gland Hypofunction in Preclinical In Vivo Models: A Systematic Review and Meta-Analysis

Paper II was a systematic review and meta-analysis investigating the safety and effect of MSC therapy for radiation-induced salivary gland damage and hypofunction in animal models. The study showed a significant increase in SFR following MSC treatment, which was the primary endpoint. Another interesting finding was that the effect on the SFR was associated with administration route, with intraglandular administration being more favorable than systemic transplantation. To elucidate the mode of action, most studies reported aspects of salivary gland remodeling following MSC treatment, including increased density of acinar cells, improved acinar structure, and reduced fibrosis. Upregulation of structure-related genes, epithelial markers and genes involved in cell migration, and cell survival and differentiation were also reported. In line with the reorganizational and regenerative properties of MSCs[117], this study reported increased vascular areas and increased paracrine functioning through several growth factors contributing to cellular proliferation, angiogenesis, and neural regeneration. These findings suggest a complex mode of action underlying MSC therapy for radiation-induced salivary gland damage and hypofunction.

The strength of this study is that it comprises a substantial sample size and included a meta-analysis of the primary endpoint, SFR. Second, a protocol article was published prior to the initiation of the study[142], for transparency and to reduce data-driven analysis. Lastly, the studies were quality evaluated according to both the ARRIVE guidelines[143] and the SYRCLE risk of bias tool[144].

The study has several limitations. Firstly, the included studies exhibited a high degree of heterogeneity with diverse methodologies. These differences encompassed variations in study design, animal models used, origin of MSCs, administration routes, radiation protocols, and follow-up regimen. Second, the RT regimens used were standardized and do not reflect the RT regimens used in head and neck cancer treatment; for example, RT was delivered as a single dose in all studies. This is important, because the results might not be translational to clinical practice. Other factors that might influence the effect of MSCs include the duration of radiation and the timing from radiotherapy to MSC therapy, both of which are challenging to translate from animal models to a clinical setting.

Paper II showed a significant therapeutic potential for MSC therapy in treating radiation-induced salivary gland damage and hypofunction, but long-term, randomized, clinical trials in humans are needed to evaluate the effect in a clinical setting.

Paper III: Long-term Effectiveness and Safety of Mesenchymal Stromal Cell Therapy for Radiation-Induced Hyposalivation in Head and Neck Cancer Survivors: A Randomized, Placebo-Controlled, Phase-2 Trial

This study represents the largest randomized trial evaluating the long-term results of the MESRIX-III clinical trial, investigating intraglandular ASCs as a treatment for radiation-induced salivary gland damage and hypofunction in head and neck cancer survivors. The primary endpoint was effect on salivary gland function, measured as UWS. Secondary aims included SWS, safety (SAEs), PROMs, and immune response (development of de novo DSAs).

The study revealed no significant difference in change in UWS between the group receiving ASCs and the group receiving placebo, with a 25% increase in the ASC group and a 27% increase in the placebo group at 12 months following intervention. This is consistent with the four-month results of the trial[128]. A significant decrease in the symptom burden related to the subjective feeling of dry mouth (EORTH-H&N35 HNDR) in the ASC group compared to placebo was observed, which was not reported at four months[128]. However, this is in line with the results from the MESRIX-I study, which reported significant long-term effect of ASCs in PROMs[131]. A decrease in the symptom burden for sticky saliva, swallowing, and xerostomia was found for ASC therapy, but with no significant differences from patients receiving placebo.

It is noteworthy that 38% (n=23) of ASC patients developed DSA, although MSCs in general are thought to be immune evasive[126]. Our findings suggest that the immune response is transient, and 70% of those who developed an immune response at four months experienced a resolved or reduced response at 12 months. A temporary immune response has also been seen in platelet transfusions[150]. The development of DSA was

associated with a reduced effect of ASCs; however, this should be interpreted cautiously due to the small sample size of the group. DSAs pose a considerable challenge in solid organ transplantation and could be relevant for a subset of patients undergoing ASC therapy to achieve therapeutic effect[151].

The study revealed an effect of placebo in all the included endpoints, both subjective and objective measurements. This could indicate a continuous natural restoration of the salivary glands beyond two years from RT, which is supported by others[152]. However, the placebo solution consisting of Cryostor10 (BiolifeSolutions) with 10% DMSO may exhibit anti-inflammatory properties acting as a therapeutic agent, as shown in other diseases[153–155]. We used ASCs from three healthy donors but observed variability in donor efficacy, with some donors producing a greater increase in UWS compared to others. Donor-variation have been reported by others[156], and while this remains speculative, it could be an important factor to consider in future trials.

The strengths of the study include the large sample size, as the study is the largest randomized trial investigating the long-term effect of ASCs for radiation-induced salivary gland damage and hypofunction. Still, this study was not conducted across multiple centers, which would have strengthened our findings. Further, the sample size was insufficient to thoroughly investigate subgroups, and larger, multicenter studies are needed to validate these findings comprising patient-specific factors such as development of DSAs, smoking, mean radiation dose, and time since radiation treatment. These studies are necessary to identify patients who may have a greater effect from ASCs, which this study was not powered to examine.

The study benefits from a randomized design, but it became unblinded after four months for both study personnel and participants, resulting in unblinded long-term follow-up. This is important to keep in mind when interpreting the results as this might overestimate the effect of ASCs and underestimate the effect of placebo, and might especially impact the PROMs.

Another strength is that the sialometry was performed as standardized as possible[128]: 1) it was performed at the same time of the day throughout the study period to minimize daily fluctuation in SFR, 2) patients were instructed to drink a minimum of two liters the day before to minimize the risk of dehydration, and 3) patients were instructed not to eat, drink, or do oral hygiene one hour before. Conversely, the sialometry evaluations were conducted only once per follow-up period, which may not fully capture the fluctuating nature of hyposalivation, which can be influenced by other factors such as stress and can vary with the seasons and room temperature[157,158]. Even though only validated questionnaires were used for evaluating PROMs, they might not encapsulate the entire picture, when collected at a single time point. Another limitation lies in the inherent nature of the PROMs. For example, the XQ covers both sleep disturbances, and difficulties eating and chewing. However, not all patients are affected by all three, but this will not be

evident in the sum scores. In addition, ASC therapy might have a larger impact on one domain than the others, but improvements in one domain might be invisible in the sum scores. No questions in the questionnaires are related to subjects like social eating, taste, or avoidance of certain foods or substances. Although these domains were frequently mentioned by the patients, we did not evaluate them. Only one question in the XQ was related to sleep disturbance but it was often reported by the patients. Lastly, the subjective importance of certain symptoms may vary among patients, as one domain might be more significant to one patient than to another; however, this variation may not be adequately captured by the questionnaires. It would be interesting to evaluate the QoL outcomes in a qualitative study, and maybe as patient-*defined* outcomes. A future approach could include a goal attainment scale, which is an individual scoring system to evaluate the extent of progress within a patient's individual goal as defined before the intervention. This method would account for variability in patients' symptoms, while emphasizing the aspects that are most important to each patient as well.

Paper IV: No Changes in the Salivary Proteome Composition Detected After Intraglandular Mesenchymal Stem Cell Therapy for Radiation-Induced Xerostomia in Previous Head and Neck Cancer Patients: A Randomized Phase 2 Trial

This study represents the largest randomized, placebo-controlled trial evaluating the mode of action of intraglandular ASCs as a treatment for radiation-induced salivary gland damage and hypofunction in head and neck cancer survivors. The primary endpoint was change in the salivary proteome composition following ASC therapy at four months compared to placebo, as evaluated by mass spectrometry analysis.

The study detected no changes in the salivary proteome following ASC therapy compared to placebo at four months. This is in line with the clinical results from the study, which did not establish superiority of ASC therapy over placebo in effect on UWS. However, we expected to see some changes as our pilot study, including 10 patients with early stage OPSCC (Stage I-II), revealed significant alterations following ASC therapy at four months compared to baseline, including upregulation of important salivary proteins involved in regeneration and immune defense, yet not restored to normal[138]. The pilot study identified significant upregulation of salivary proteins in healthy controls compared to irradiated patient. We did observe a non-significant upregulation of most of these proteins following ASCs compared to placebo (e.g., cystatins and Lipocalin-1) indicating a trend towards a healthier saliva proteome composition following ASCs and partial repair. These proteins are, among others, important for the overall oral health and taste perception [159].

The increase in the clinical outcome UWS was associated with smoking, higher mean radiation dose to the four large salivary glands and development of DSAs at four months[128]. Therefore, we hypothesized that an impact on the salivary proteome would be more prominent in the subgroups where we expected the highest effect of ASCs; that is among never smokers, patients who received a lower mean radiation dose (40 Gy), patients who did not develop DSAs, and those who experienced a clinical effect (>30% increase in UWS). No changes in the salivary proteome were detected among these subgroups, but a tendency towards upregulation of salivary proteins upregulated in healthy saliva was observed in those who did not develop DSAs. This indicates that immunological tolerance might be important to restore the saliva composition.

We compared the irradiated salivary proteome with healthy controls. Enriched biological terms in the irradiated proteome comprised an increased metabolic rate, reflecting the metabolic processes necessary in saliva homeostasis, but also possibly due to a higher need to repair chronic radiation damage. RT leads to oxidative stress[160], which was enriched in irradiated patients. Increased oxidative stress and inflammation in saliva from irradiated patients have been reported by others[139,140]. Interestingly, the study revealed that the salivary proteome in patients who received a lower mean radiation (<40 Gy) was associated with significant upregulation of Cystatin-S, -SN and -SA and Liocalin-1, which are upregulated in healthy saliva[138,161]. This points out the importance of minimizing radiation dose to protect the salivary glands to improve both the salivary flow rates and the salivary quality.

The study benefits from a randomized design and the large sample size. The sialometry was conducted as standardized as possible as described for Paper III, but we only collected the saliva samples once per follow-up visit, which is a limitation of our study. Other limitations include a high proportion of excluded samples, with significantly more smokers in the excluded population compared to the included population. Smoking negatively impacted the clinical effect on UWS[128], and could influence the effect on the salivary proteome composition. However, we did not observe any differences in never smokers in the ASC group at four months compared to baseline or in the irradiated patients with a history of smoking compared to never smokers.

Our study population was heterogenous, encompassing various subsites, differing radiation doses, and varying time intervals since radiation exposure. Additionally, there was significant interpersonal variability in salivary proteins levels. This reduces the likelihood of identifying small changes caused by ASC therapy. In line with this, the study was powered to identify an effect in the clinical outcomes UWS, and not changes in the salivary proteome. Lastly, we compared unstimulated salivary samples from irradiated patients with stimulated salivary samples in healthy controls, which have been shown to differ[162,163]. For example, the

metabolic rate is higher in stimulated saliva, but our results indicated a higher metabolic rate in unstimulated saliva from irradiated patients, and this difference may be even more prominent.

Conclusion and perspectives

To advance treatment stratification and rehabilitation of head and neck cancer patients in the era of HPV, understanding the nuanced interplay of patient specific factors, oncological outcomes, and recovery is essential. This thesis seeks to address distinct but interconnected aspects of these domains, focusing on the clinical, treatment, and prognostic differences in diverse HPV+ OPSCC populations (**Paper I**), the potential of ASC therapy in regenerative medicine for radiation-induced salivary gland damage and hypofunction in animal models (**Paper II**), and the long-term effect and mode of action of intraglandular ASC therapy in alleviating radiation-induced salivary gland damage and hypofunction, and dry mouth in head and neck cancer survivors (**papers III and IV**).

In **Paper I** we investigated to what extent HPV+ OPSCC populations differ in the largest retrospective cohort study comparing European and American high HPV-prevalence OPSCC populations from diverse healthcare systems. The study comprised patients from the highest-ranked cancer center in in the U.S, UTMDACC, and from Eastern Denmark, representing an insurance-based and a universal, public healthcare system, respectively. We found significant differences in demographics, clinical characteristics, and treatment regimen used. While the three-year overall survival was comparable, the Copenhagen cohort exhibited a higher risk of recurrence. These findings highlight critical clinical, therapeutic, and prognostic differences between a Northern European and a North American OPSCC population. Such variations must be taken into account when comparing outcomes and stratifying patients in clinical trials to escalated or de-escalated therapy. To advance treatment stratification in clinical trials and future clinical practice, it is essential to explore these inter-cohort differences. This will help ensure that results from clinical studies are globally applicable across diverse OPSCC populations and healthcare systems. Multicenter studies are needed to thoroughly understand these transnational differences, and between OPSCC cohorts from healthcare systems that are more alike. To investigate this further, our research group has just initiated a Nordic collaboration with centers in Sweden (Karolinska, Uppsala and Lund University Hospitals), Finland (Helsinki University Hospital), Norway (Bergen University Hospital), and Iceland (Landspitali University Hospital) all operating within universal, public healthcare systems and with unique personal identification numbers. Important strengths of such a collaboration are the long follow-up available within such a healthcare setup and the availability of double HPV/p16 status. Multiple studies are in the pipeline but comprise, among others, a similar comparison of clinical, treatment, and prognostic differences, examination of the long-term prognosis with focus on the risk of late recurrences, and the use of HPV/p16 status in other oropharyngeal subsites.

Given that the incidence of HPV+ OPSCC continues to increase[20] and is expected to remain high for many years to come, despite effective HPV vaccination, due to the long incubation period of HPV infections, ongoing research to optimize treatment strategies is crucial. It is essential to identify HPV+ OPSCC patients at higher risk of severe disease progression and gain a deeper understanding of the mutational profile in those who develop distant metastases. A distinct mutational profile has been found in HPV+ OPSCC with recurrent disease compared to HPV+ OPSCC without recurrent disease[164]. Although this has not yet been validated in larger studies, mutational profiling might be useful in identifying the subgroup with aggressive, metastasizing HPV+ OPSCCs that would not benefit from de-escalated treatment. Our research group has started a consecutive, population-based study focused on the years from 2000-2020 with the aim of evaluating the mutational profile (Regional Scientific Ethical Committee H-230476672) in HPV+ OPSCCs with lung metastasis. Additionally, early detection of recurrence is important to increase the chances of curative treatment. To improve the ability to detect recurrent disease, a prospective, randomized trial investigating the use of ctHPV DNA, as part of the follow-up regimen, has been initiated (Regional Scientific Ethical Committee H-23071576), based on the promising results from a pilot study conducted at Rigshospitalet[72]. Furthermore, to enable more patients to receive TORS as the primary treatment modality, a feasibility study investigating the use of neoadjuvant chemotherapy aimed at tumor reduction prior to TORS, will be initiated in early 2025. It is expected that the study will include patients with larger primary HPV+ OPSCC tumors, T2-T3 (N0-N1), that would not otherwise be eligible for TORS. In the long run, this might pave the way for a randomized trial in a Danish setting.

In **Paper II**, we investigated the potential of MSC therapy to restore the salivary gland function following RT in animal models. The study revealed a significant effect of MSC therapy in increasing the SFR and remodeling salivary gland tissue. These findings suggest that MSC therapy has therapeutic potential for radiation-induced salivary gland damage and hypofunction. However, to validate these results and assess the efficacy in clinical practice, human randomized clinical trials are needed.

In **Paper III**, we investigated the long-term effect and safety of intraglandular ASC treatment for radiation-induced salivary gland damage and hyposalivation. The study showed that intraglandular ASC therapy significantly reduced the subjective feeling of dry mouth compared to placebo. Although both the ASC and placebo groups demonstrated increases in UWS, there was no significant objective improvement in salivary gland function with ASC therapy compared to placebo. ASC therapy may only benefit a subset of patients since effect on UWS was associated with mean radiation dose, smoking, and development of DSAs. This suggests that although intraglandular ASC therapy may alleviate the sensation of dry mouth, its impact on objectively restoring salivary gland function remains inconclusive and warrants further investigation in a

multicenter setting and powered to investigate the effect in subgroups. A more qualitative approach to evaluation of PROMs might reveal improvements in domains not fully elucidated in the questionnaires included in this study, such as sleep disturbances, while also considering the patients' unique challenges.

The previous MESRIX-I study utilized MRIs and salivary biopsies to assess the mode of action, but these methods were not incorporated in this study due to practical and ethical considerations[130]. As a mode of action, The MESRIX-II study examined the salivary proteome and identified upregulation of proteins associated with regeneration[138], and this approach was incorporated in the MESRIX-III trial, **Paper IV**. The study did not detect changes in the salivary proteome composition following ASC therapy compared to placebo, although several important salivary proteins upregulated in healthy saliva were non-significantly upregulated the ASC group, indicating an incomplete restoration. Future studies on the salivary composition and salivary proteome are valuable, as ASCs may not only enhance saliva production but improve saliva quality as well.

In multiple sclerosis and osteoarthritis, repeated administrations with MSCs have shown beneficial results[165,166]. This has not yet been investigated in radiation-induced hyposalivation and may be favorable as well. A randomized study addressing repeated treatment of patients who previously participated and received ASCs in our MESRIX-I, -II, and -III studies is expected to begin primo 2025. Additionally, ASC treatment may be beneficial in xerostomia and hyposalivation caused by inflammatory disease, such as Sjögrens Syndrome[167], which we will investigate in a randomized, phase 2 study scheduled to begin early 2025. Finally, intraglandular ASC therapy has not yet been validated in a large, multicenter setting, which is necessary to identify subgroups that may have an improved effect. We expect to conduct such a multicenter study in collaboration with the Karolinska University Hospital in the near future.

First and co-authorships in HPV, head and neck cancer, and mesenchymal stem cells

Papers 1-3 are included in the thesis.

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Appendix I – 8th edition of the TNM AJCC/UICC staging manual for OPSCC[53]

p16+ OPSCC

T category	N category	M category	Overall stage
T0, T1 or T2	N0 or N1	M0	I
T0, T1 or T2	N2	M0	II
T3	N0, N1 or N2	M0	II
T0, T1, T2, T3 or T4	N3	M0	III
T4	N0, N1, N2 or N3	M0	III
Any T	Any N	M1	IV

p16- OPSCC

T category	N category	M category	Overall stage
Tis	N0	M0	0
T1	N0	M0	I
T2	N0	M0	II
T3	N0	M0	III
T1, T2 or T3	N1	M0	III
T4a	N0 or N1	M0	IVA
T T1, T2, T3 or T4a	N2	M0	IVA
Any T	N3	M0	IVB
T4b	Any N	M0	IVB
Any T	Any N	M1	IVC

Abbreviations: AJCC, American Joint Committee on Cancer; M, metastases; N, nodes; p16, p16^{Ink4a} tumor suppressor protein; T, tumor; UICC, Union of International Cancer Control.

Appendix II – Xerogenic medicine[168]

Anatomical group	Therapeutic group	Chemical substance
Alimentary tract/metabolism	Anticholinergics	Propantheline Atropine Scopolamine
Cardiovascular system	Antihypertensives	Clonidine Methyldopa
	Diuretics	Bendroflumethiazide Furosemide Tolvaptan
	Beta-blockers	Timolol Metoprolol Atenolol
	Calcium channel blockers	Isradipine Verapamil
	ACE inhibitors	Enalapril Lisinopril
Musculoskeletal system	Bisphosphonates	Alendronate
Nervous system	Analgesics	Fentanyl Morphine Buprenorphine Butorphanol Tramadol Clonidine
	Antipsychotics	Chlorpromazine Haloperidol Clozapine Olanzapine Quetiapine Lithium

		Risperidone Zolpidem
	Antidepressants	Amitrityline Citalopram Sertraline Escitalopram Venflaxine Duloxetine Bupropion
Respiratory system	Drugs for obstructive airway diseases	Tiotropium
	Antihistamines	Cetirizine Doxylamine
Sensory organs	Ophthalmologicals	Atropine Brimonidine

*Modified table from Wolff et al., Drug R D, 2017[168]

Abbreviations: ACE, angiotensin-converting enzyme.

Appendix III – EORCT QLQ H&N35

DANISH

EORTC QLQ - H&N35

Patienter fortæller undertiden, at de har følgende symptomer eller problemer. Anfør venligst, i hvilket omfang De har haft disse symptomer eller problemer inden for den forløbne uge. Besvar spørgsmålene ved at sætte en ring omkring det tal, som passer bedst til Dem.

I den forløbne uge:		**Slet ikke**	**Lidt**	**En del**	**Meget**
31.	Har De haft smerter i munden?	1	2	3	4
32.	Har De haft smerter i kæben?	1	2	3	4
33.	Har De været øm i munden?	1	2	3	4
34.	Har De haft ondt i halsen?	1	2	3	4
35.	Har De haft svært ved at synke væske?	1	2	3	4
36.	Har De haft svært ved at synke most, blendet eller pureret mad?	1	2	3	4
37.	Har De haft svært ved at synke fast føde?	1	2	3	4
38.	Har De fået noget galt i halsen, når De har sunket?	1	2	3	4
39.	Har De haft problemer med tænderne?	1	2	3	4
40.	Har De haft svært ved at åbne munden helt?	1	2	3	4
41.	Har De været tør i munden?	1	2	3	4
42.	Har Deres spyt virket klæbende?	1	2	3	4
43.	Har De haft problemer med lugtesansen?	1	2	3	4
44.	Har De haft problemer med Deres smagssans?	1	2	3	4
45.	Har De hostet?	1	2	3	4
46.	Har De været hæs?	1	2	3	4
47.	Har De følt Dem syg?	1	2	3	4
48.	Har Deres udseende generet Dem?	1	2	3	4

Gå venligst videre til næste side

I den forløbne uge:		Slet ikke	Lidt	En del	Meget
49.	Har De haft svært ved at spise?	1	2	3	4
50.	Har De haft svært ved at spise i Deres families nærvær?	1	2	3	4
51.	Har De haft svært ved spise i andre folks nærvær?	1	2	3	4
52.	Har De haft svært ved at nyde Deres måltider?	1	2	3	4
53.	Har De haft svært ved at tale til andre?	1	2	3	4
54.	Har De haft svært ved at tale i telefon?	1	2	3	4
55.	Har De haft svært ved at være sammen med Deres familie?	1	2	3	4
56.	Har De haft svært ved at være sammen med venner?	1	2	3	4
57.	Har De haft svært ved at gå ud blandt andre?	1	2	3	4
58.	Har De haft svært ved at have fysisk kontakt med familie eller venner?	1	2	3	4
59.	Har De haft mindre lyst til seksuelt samvær?	1	2	3	4
60.	Har De følt mindre seksuel nydelse?	1	2	3	4

I den forløbne uge:		Nej	Ja
61.	Har De taget smertestillende medicin?	1	2
62.	Har De taget nogen form for kosttilskud (bortset fra vitaminpiller)?	1	2
63.	Har De anvendt en ernæringssonde?	1	2
64.	Har De tabt Dem?	1	2
65.	Har De taget på?	1	2

Appendix IV – Xerostomia Questionnaire

Confidential

XQ-spørgeskema

Please complete the survey below.

Thank you!

1) REDCap username _____

	Ingen problemer 0	1	2	3	4	5	6	7	8	9	Kunne ikke være værre/ værst tænkelig tørhed 10
2) 1. Angive hvor svært du har ved at tale på grund af mundtørhed	○	○	○	○	○	○	○	○	○	○	○
3) 2. Angiv hvor svært du har ved at tygge på grund af mundtørhed	○	○	○	○	○	○	○	○	○	○	○
4) 3.Angiv hvor svært du har ved at synke faste fødevarer på grund af mundtørhed	○	○	○	○	○	○	○	○	○	○	○
5) 4. Angiv hvor ofte du har søvnproblemer på grund af mundtørhed	○	○	○	○	○	○	○	○	○	○	○
6) 5. Angiv hvor tør du er i mund eller svælg, når du spiser mad	○	○	○	○	○	○	○	○	○	○	○
7) 6. Angiv hvor tør du er i mund eller svælg, når du ikke spiser	○	○	○	○	○	○	○	○	○	○	○
	Aldrig 0	1	2	3	4	5	6	7	8	9	Altid 10
8) 7. Angiv hvor ofte du har brug for at nippe til væske for at synke mad	○	○	○	○	○	○	○	○	○	○	○
9) 8. Angiv hvor ofte du nipper væske for lindring i munden, når du ikke spiser	○	○	○	○	○	○	○	○	○	○	○

Appendix V – Scientific papers I-IV

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Clinical and prognostic differences in oropharyngeal squamous cell carcinoma in USA and Denmark, two HPV high-prevalence areas

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ABSTRACT

Background: Uncertainty persists regarding clinical and treatment variations crucial to consider when comparing high human papillomavirus (HPV)-prevalence oropharyngeal squamous cell carcinoma (OPSCC) cohorts for accurate patient stratification and replicability of clinical trials across different geographical areas.
Methods: OPSCC patients were included from The University of Texas MD Anderson Cancer Center (UTMDACC), USA and from The University Hospital of Copenhagen, Denmark from 2015–2020, (n = 2484). Outcomes were 3-year overall survival (OS) and recurrence-free interval (RFI). Subgroup analyses were made for low-risk OPSCC patients (T1–2N0M0) and high-risk patients (UICC8 III-IV).
Results: There were significantly more HPV-positive (88.2 % vs. 63.1 %), males (89.4 % vs. 74.1 %), never-smokers (52.1 % vs. 23.7 %), lower UICC8-stage (I/II: 79.3 % vs. 68 %), and fewer patients treated with radiotherapy (RT) alone (14.8 % vs. 30.3 %) in the UTMDACC cohort. No difference in the adjusted OS was observed (hazard ratio [HR] 1.21, p = 0.23), but a significantly increased RFI HR was observed for the Copenhagen cohort (HR: 1.74, p = 0.003). Subgroup analyses of low- and high-risk patients revealed significant clinical and treatment differences. No difference in prognosis was observed for low-risk patients, but the prognosis for high-risk patients in the Copenhagen cohort was worse (OS HR 2.20, p = 0.004, RFI HR 2.80, p = 0.002).
Conclusions: We identified significant differences in clinical characteristics, treatment modalities, and prognosis between a Northern European and Northern American OPSCC population. These differences are important to consider when comparing outcomes and for patient stratification in clinical trials, as reproducibility might be challenging.

1. Introduction

Human papillomavirus (HPV) is a key factor in the rising incidence of oropharyngeal squamous cell carcinoma (OPSCC), but with geographical variation [1–4]. How HPV-positivity is defined varies, but the expression of p16 is widely accepted as a surrogate marker for HPV-positivity. However, double p16/HPV-positivity more accurately classify biologically active HPV infection, better predicts patient

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outcomes, and standardized HPV testing has been recommended [4,5].

Given the remarkable treatment response [6] and the toxicities associated with treatment [7], attempts have been made to identify low-risk HPV-positive (HPV+) patients suitable for treatment de-intensification [8,9]. Some well described factors associated with low-risk OPSCC include limited smoking history, p16 + status, and low tumor burden [6].

Both the U.S. and Denmark are HPV high-prevalence areas [10,11]. However, the healthcare systems in Denmark and the U.S. exhibit significant differences. Denmark operates under a public, universal healthcare system and all cancer diagnoses, treatments, and follow-ups are conducted with the standardized cancer care packages available within the public health care system. The healthcare system in the U.S. is larger and more complex, with patients being treated at private or public care centers based on a variety of factors, including access to health insurance.

The consideration of potential clinical and demographic variations is crucial when interpreting the prognosis across different patient populations, for accurate patient stratification and management and to ensure reproducibility and replicability of clinical trials in diverse geographical areas.

The aim of this study was to identify potential differences in clinical characteristics, treatment modalities given, and prognosis among OPSCC patients in two HPV high-prevalence areas with distinct populations and health care systems: The Copenhagen Oropharyngeal Cancer Database (COHOC) at the University Hospital of Copenhagen, Denmark and the Stiefel OPSCC Database at The University of Texas MD Anderson Cancer Center, USA (UTMDACC). Second, we wished to identify if such differences impact the prognosis for a subgroup of low-risk (T1T2N0M0) and high-risk (III-IV) patients potentially eligible for de-escalated or intensified therapy, respectively.

2. Materials and methods

2.1. Study design, setting and population

This retrospective cohort study was approved by The Regional Scientific Ethical Committee (H-20072877) and the Danish Data Protection Agency and followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline [12]. The Stiefel OPSCC Database is approved by The Institutional Review Board of the University of Texas MD Anderson Cancer Center (UTMDACC) (protocol PA 14–0947).

The study cohort consisted of all patients with a new OPSCC diagnosed from 2015–2020 treated with curative intent from two independent OPSCC cohorts: *The COHOC Database* is a population-based, retrospective cohort, comprising patients diagnosed with OPSCC from 2000–2020 in Eastern Denmark and was previously well described [13–15]. *The Stiefel OPSCC Database* is a prospective, longitudinal cohort initiated at UTMDACC. Since 2015, 1671 OPSCC patients have been enrolled with detailed characterization of exposures, HPV-status, diagnostic/staging, treatment, disease control, and longitudinal collection of validated clinician- and patient-reported survivorship outcomes from diagnosis through 5-years of follow-up.

2.2. Variables

The primary outcomes were 3-year overall survival (OS) and 3-year recurrence-free interval (RFI). OS was defined as time from diagnosis to death of any cause and patients alive were censored at the last date of follow-up or 3 years after diagnosis. RFI was defined as the time from primary diagnosis to the diagnosis of recurrence (locoregional and/or distant) and patients without recurrence were censored at last day of follow-up, date of death or 3-years after diagnosis. HPV-positivity was defined as being positive for both HPV DNA and p16 in the Copenhagen cohort, while HPV-positivity was defined as being positive to HPV or

Table 1
Characteristics of 2484 patients with OPSCC in Eastern Denmark and UTMDACC, USA from 2015–2020.

Variable	Eastern Denmark. n = 1216		UTMDACC, USA. n = 1268		
	no.	%	no.	%	*P-value*[a]
Gender					< *0.001*[a]
Male	901	74.1	1133	89.4	
Female	315	25.9	135	10.6	
Median age (IQR)	62 (56-70)		60 (54-67)		< *0.001*[a]
no.	1216		1268		
Median follow-up. years (IQR)	2.3 (1.7-3.3)		2.1 (1-3.6)		< *0.001*[a]
no.	1216		1210		
Smoking					< *0.001*[a]
Current	464	38.2	66	5.2	
Former	458	37.7	535	42.2	
Never	288	23.7	661	52.1	
Unknown	6	0.5	6	0.5	
HPV-status					< *0.001*[a]
HPV+	767	63.1	1119	88.2	
HPV-	420	34.5	98	7.7	
Unknown	29	2.4	51	4	
HPV/p16-status					< *0.001*[a]
HPV+/p16+	767	63.1	345	27.2	
HPV+/p16-	49	4.0	8	0.6	
HPV-/p16+	35	2.9	18	1.4	
HPV+/p16-	49	4.0	8	0.6	
HPV-/p16-	327	26.9	23	1.8	
Unknown	38	3.1	874	68.9	
HPV genotype					< *0.001*[a]
HPV16	662	87.8	109	87.9	
HPV33	59	7.8	1	0.87	
Other genotypes	33	4.4	14	11.3	
Tumor location					< *0.001*[a]
BOT	374	30.8	617	48.7	
Tonsil	628	51.6	599	47.2	
Other	214	17.6	52	4.1	
TNM Stage (UICC/AJCC 8), HPV+ T-class					*0.005*[a]
T1	241	31.4	400	35.7	
T2	289	37.7	433	38.7	
T3	116	15.1	168	15.0	
T4	119	15.5	118	10.5	
Unkown	2	0.3	0		
N-class					< *0.001*[a]
N0	121	15.8	125	11.2	
N1	514	67.0	768	68.6	
N2	103	13.4	203	18.1	
N3	29	3.8	23	2.1	
Unknown	0		0		
M-class					*0.01*[a]
M0	762	99.3	1093	97.7	
M1	2	0.3	14	1.8	
Unknown	3	0.4	12	1.1	
Overall Stage (UICC/AJCC 8), HPV+					*0.001*[a]
I	462	60.2	709	63.4	
II	220	28.7	259	23.1	
III	81	10.6	124	11.1	
IV	1	0.1	15	1.3	
Unknown	3	0.4	12	1.1	
TNM Stage (UICC/AJCC 8), HPV- T-class					
T1	105	25.0	30	30.6	*0.74*
T2	138	32.9	32	32.7	
T3	92	21.9	20	20.4	
T4	84	20.0	16	16.3	

(*continued on next page*)

Table 1 (*continued*)

Variable	Eastern Denmark. n = 1216		UTMDACC, USA. n = 1268		
	no.	%	no.	%	*P-value*[a]
Unknown	1	0.2	0		
N-class					*0.001*[a]
N0	162	38.6	21	21.4	
N1	76	18.1	34	34.7	
N2	156	37.1	37	37.8	
N3	24	5.7	6	6.1	
Unknown	2	0.5	0		
M-class					*0.001*[a]
M0	412	98.1	93	94.9	
M1	6	1.4	1	1.0	
Unknown	2	0.5	4	4.1	
Overall Stage (UICC/AJCC 8), HPV-					*0.22*
I	74	17.6	18	18.4	
II	71	16.9	9	9.2	
III	72	17.1	18	18.4	
IV	198	47.1	50	51.0	
Unknown	5	1.2	3	3.1	
Treatment					< *0.001*[a]
RT	368	30.3	188	14.8	
CRT	620	51.0	631	49.8	
Neoadjuvant chemotherapy + RT/CRT	-	-	212	16.7	
Surgery	195	16.0	110	8.7	
Surgery + RT/CRT	33	2.7	124	9.8	
Systemic therapy alone	0	-	2	0.2	
Unspecified curative treatment	0	-	1	0.1	

Note: Frequency (%) is provided for categorical variables, median (IQR) are provided for continuous variables. Chi Square test were used for categorical variables, while t-test was used for continuous variables. Fisher's exact test was used for small sample sizes. *Abbreviations:* UTMDACC, The University of Texas MD Anderson Cancer Center, USA; IQR, interquartile range; HPV, human papillomavirus; BOT, base of tongue; RT, radiotherapy; CRT, chemoradiotherapy.

[a] Significant value.

p16 in the UTMDACC cohort. Details on HPV detection, p16 immunohistochemistry, and measurements are provided in Supplement 1.

2.3. Treatment

Therapeutic decisions were made during a multidisciplinary treatment planning conference based on factors such as disease stage, anatomical subsite, comorbidity, functional considerations, and the preferences of both patients and clinicians. In Denmark, treatment decisions are in accordance with the Danish Head and Neck Cancer Group (DAHANCA) national guidelines [16,17], while treatment decisions generally follow the National Comprehensive Cancer Network (NCCN) guidelines at UTMDACC [18]. Details on treatment guidelines are provided in Supplement 1.

2.4. Statistics

Statistical analysis was performed in R statistics version 4.1.3. Continuous variables were reported as median values with an interquartile range (IQR) and categorical variables as frequencies. To test for significance, Pearson's chi-square test was used for the binomial categorical covariables, Fisher's exact test was used for small sample sizes, and a *t*-test was used for the quantitative covariables distributed in two groups. We considered a p-value < 0.05 to be statistically significant. Age and follow-up time were included as continuous variables and the other variables were considered categorical variables.

OS and RFI were evaluated by Kaplan–Meier curves and with the log-rank method and by uni- and multivariable Cox regression analyses (packages Survminer and Survival). For the total OPSCC cohort, the OS (372 events) and RFI (267 events) multivariable analyses were adjusted for treatment center, age (cut-off >60 years), gender, HPV-status, smoking status, tumor location, T-, N- and M-class and treatment modality. For model control, proportionality was tested with Score residuals. Details on subgroup analyses are provided in Supplement 1.

3. Results

3.1. Patient characteristics

A total of 2484 patients were included, 1216 patients from Eastern Denmark and 1268 from UTMDACC. The median follow-up time was 2.1 years (IQR:1.0–3.6) in the UTMDACC cohort and 2.3 years (IQR: 1.7–3.3) in the Copenhagen cohort, $p < 0.001$. The UTMDACC cohort included significantly more men (89.4 % vs. 74.1 %), younger age (median age 60 vs. 62 years), and never smokers (52.1 % vs. 23.7 %). See Table 1.

3.2. Clinical tumor characteristics

In the UTMDACC cohort, the predominant tumor site was the base of

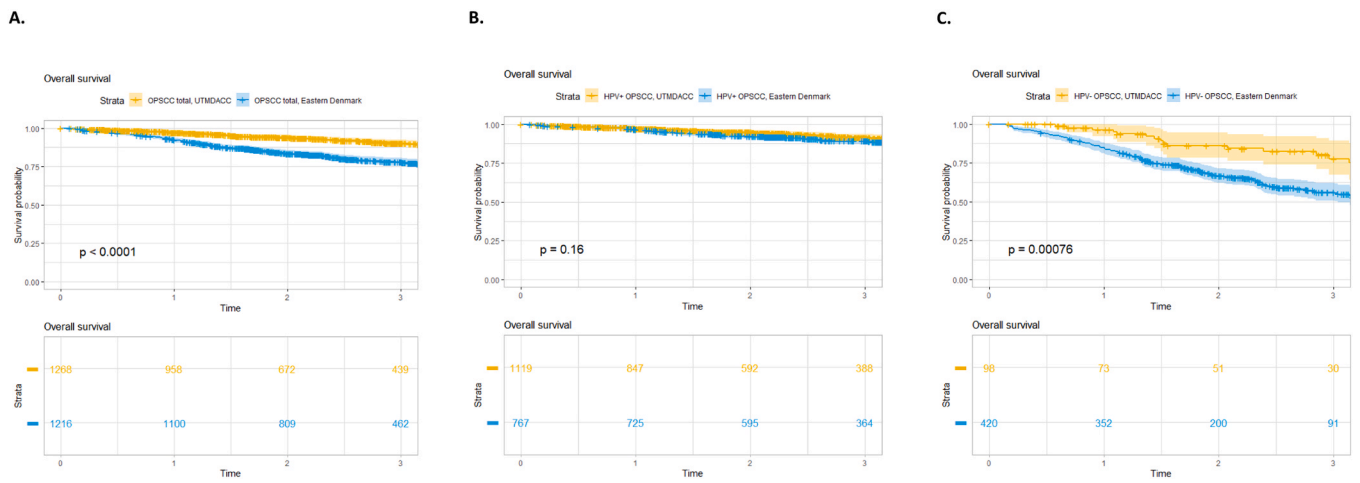

Fig. 1. Kaplan-Meier curves depicting the overall survival probability stratified by center and HPV status from 2015–2020. A. All OPSCC 2015–2020 stratified by center. B. HPV+ OPSCC stratified by center. C. HPV- OPSCC stratified by center. *Abbreviations:* UTMDACC, The University of Texas MD Anderson Cancer Center, USA, HPV, human papillomavirus; OPSCC, oropharyngeal squamous cell carcinoma.

Table 2
Multi- and univariable analysis for overall survival for OPSCC in Eastern Denmark and UTMDACC from 2015–2020.

	Univariable			Multivariable[c]		
Variable	HR	95 % CI	p-value[c]	HR	95 % CI	p-value[b]
Center (UTMDACC ref)						
Eastern Denmark	2.20	1.77-2.75	< 0.001[b]	1.21	0.89-1.65	0.23
Age (<60 years ref)> 60 years	1.87	1.51-2.32	< 0.001[b]	1.22	0.97-1.55	0.09
Gender (female ref)						
Male	1.02	0.79-1.32	0.90	1.30	0.99-1.72	0.06
HPV status (HPV+ ref)						
HPV-	4.93	4.00-6.07	< 0.001[b]	2.56	1.92-3.41	< 0.001[b]
Smoking status (never ref)						
previous	1.78	1.33-2.39	< 0.001[b]	1.31	0.96-1.79	0.09
current	5.08	3.83-6.73	< 0.001[b]	2.26	1.58-3.22	< 0.001[b]
Tumor location (tonsil ref)						
BOT	0.97	0.76-1.22	0.77	0.95	0.74-1.22	0.69
Other	2.62	2.02-3.41	< 0.001[b]	0.86	0.64-1.16	0.33
T-class (T1 ref)						
T2	1.42	1.04-1.93	0.03[b]	1.28	0.93-1.77	0.14
T3	3.37	2.46-4.61	< 0.001[b]	2.63	1.86-3.71	< 0.001[b]
T4	3.87	2.84-5.29	< 0.001[b]	2.52	1.78-3.57	< 0.001[b]
N-class (N0 ref)						
N1	0.48	0.36-0.64	< 0.001[b]	1.17	0.85-1.65	0.34
N2	1.60	1.21-2.12	0.001[b]	1.88	1.38-2.57	< 0.001[b]
N3	2.92	1.94-4.39	< 0.001[b]	3.79	2.42-5.95	< 0.001[b]
M-class (M0 ref)						
M1	3.93	2.26-6.84	< 0.001[b]	4.06	2.26-7.30	< 0.001[b]
Treatment regimen (CRT ref)						
RT	2.7	2.15-3.39	< 0.001[b]	2.21	1.71-2.86	< 0.001[b]
Neoajduvant chemotherapy + RT/CRT	1.49	1.04-2.15	0.03[b]	1.31	0.83-2.07	0.24
Surgery	0.70	0.46-1.07	0.10	1.24	0.78-1.98	0.36
Surgery + RT/CRT	0.56	0.31-1.01	0.05	0.89	0.48-1.65	0.71

Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; ref, reference; HPV, human papillomavirus, BOT, base of tongue; RT, radiotherapy; CRT, chemoradiotherapy.

[b] Significant value.

[c] Adjusted for center, age group, gender, HPV status, smoking status, T-site location, stage UICC8 and treatment regimen.

tongue (BOT) (48.6 %) followed by the palatine tonsils (47.2 %) while the palatine tonsils were the predominant tumor site in the Copenhagen cohort (51.6 %). OPSCC patients presented at the UTMDACC at significantly lower T-stage (T1-T2: 72.9 % vs. 64.9 %), lower N-stage (N0-N1: 76.8 % vs. 73.3 %) and lower UICC stage (I-II: 78.4 % vs. 69.2 %) than in Copenhagen. See Table 1.

3.3. HPV status

Overall, 63.1 % of the OPSCC patients were HPV+ in the Copenhagen cohort, while 88.2 % were HPV+ in the UTMDACC cohort. In the UTMDACC cohort, 68.9 % did not have both HPV and p16 status available, while this was the case in 3.1 % of the patients in the Copenhagen cohort. In the Copenhagen cohort, 0.4 % and 10.8 % in the UTMDACC were tested for HPV DNA only, while 1.9 % in the Copenhagen cohort and 46.7 % in the UTMDACC cohort were tested for p16 only. See Table 1.

3.4. Treatment modalities

Radiation-based treatment was predominant in both cohorts, but significantly more patients received radiotherapy (RT) alone in the Copenhagen cohort (30.3 % vs. 14.8 %). In the Copenhagen cohort, 51.0 % received concurrent chemotherapy (CRT) vs. 49.8 % in the UTMDACC cohort. Neoadjuvant chemotherapy + RT/CRT was given to 16.7 % of patients in the UTMDACC cohort. Significantly more patients received surgery alone in the Copenhagen cohort (16.0 % vs. 8.7 %), while significantly more received surgery and post-operative adjuvant therapy in the UTMDACC cohort (9.8 % vs. 2.7 %). See Table 1.

3.5. Overall survival

Kaplan-Meier OS models showed significant differences in the 3-year OS for the UTMDACC cohort (91 % [95 % CI: 89–93 %]) compared to the Copenhagen cohort (83 %, [95 % CI: 80–85 %], log-rank $p < 0.001$) which was also noted for HPV-negative (HPV-) patients. See Fig. 1 and supplementary Table 2 (Supplement 1).

The multivariable OS analysis revealed no significant difference between the two cohorts (hazard ratio [HR] 1.21, $p = 0.23$). A significantly increased HR was observed for HPV-, current smoking, T3- and T4-class, N2 and N3-class, M1-class, and RT. See Table 2.

3.6. Recurrence-free interval

Overall, 83 OPSCC patients had a recurrence in the UTMDACC cohort, with a median time to recurrence of 1.00 years (IQR: 0.63–1.91 years). In the Copenhagen cohort, 184 had a recurrence with a median time to recurrence of 1.04 years (IQR: 0.69–1.61). The 3-year RFI was significantly better in the UTMDACC cohort (91.3 % [95 % CI 89.3–93.3 %]) compared to the Copenhagen cohort (82.7 % [95 % CI 80.3–85.1 %], log-rank $p < 0.001$), which was also found when stratifying for HPV-status. See Fig. 2.

The multivariable recurrence analysis showed a significantly increased HR in the Danish cohort (HR: 1.74, $p = 0.003$). Also, HPV-, T2-T4-class, N1-N3-class, M1-class, RT and neoadjuvant chemotherapy + RT/CRT had a significantly increased HR. See Table 3. The analysis was also performed for patients with either progression or second primary tumors, which did not significantly impact the conclusions; data not shown.

3.7. Subgroup analysis of low-risk patients with T1/T2N0M0 OPSCC

The UTMDACC T1T2N0M0 cohort exhibited significantly more men, younger ages, more never smokers, more HPV+, fewer OPSCC from other locations, and more received CRT than the Danish T1T2N0M0 cohort. See Table 3 (Supplement 1). In total, 26 had a recurrence with a median time to recurrence of 1.07 years (IQR 0.82–1.47) and 0.76 years (IQR 0.63–1.75) in the Copenhagen and the UTMDACC T1T2N0M0 cohorts, respectively.

Kaplan-Meier models showed a significantly higher 3-year OS in the UTMDACC T1T2N0M0 cohort (89.9 % vs. 79.3 %, log-rank $p = 0.01$) and 3-year RFI (96.2 % vs. 86.5 %, log-rank $p = 0.011$). See Fig. 1 (Supplement 1). These differences were not reproduced in the

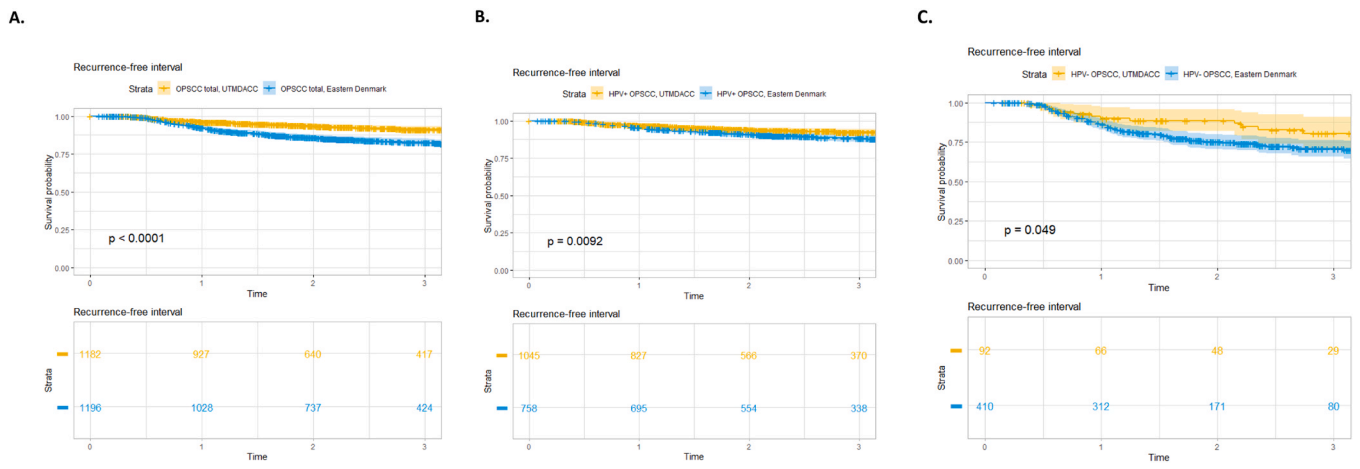

Fig. 2. Kaplan-Meier curves depicting the recurrence-free interval stratified by center and HPV status from 2015–2020. A. All OPSCC 2015–2020 stratified by center. B. HPV+ OPSCC stratified by center. C. HPV- OPSCC stratified by center. *Abbreviations:* UTMDACC, The University of Texas MD Anderson Cancer Center, USA, HPV, human papillomavirus; OPSCC, oropharyngeal squamous cell carcinoma.

multivariable analysis for both OS and RFI. See Table 4 and 5 (Supplement 1).

3.8. Subgroup analysis of high-risk patients with stage III-IV OPSCC

The UTMDACC III-IV cohort exhibited significantly more men, younger age, more never smokers, more HPV+, fewer other OPSCC locations, higher UICC-stage for HPV+ and fewer received RT. See Table 6 (Supplement 1). In total, 126 had a recurrence with a median time to recurrence of 0.88 years (IQR 0.59–1.32) and 1.19 (IQR: 0.54–1.71) in the Copenhagen and the UTMDACC III-IV cohorts, respectively.

Kaplan-Meier models showed a significantly higher 3-year OS in the UTMDACC III-IV cohort (77.1 % vs. 52.4 %, log-rank $p < 0.001$) and 3-year RFI (78.6 % vs. 65 %, log-rank $p = 0.001$). See Fig. 2 (Supplement 1). These differences were reproduced in the multivariable analysis for both OS (HR 2.20, $p = 0.004$) and RFI (HR 2.80, $p = 0.002$). See Table 7 and 8 (Supplement 1).

3.9. Subgroup analysis of RT single modality patients

See Results (Supplement 1).

4. Discussion

This study, which included 2484 patients with OPSCC from Eastern Denmark and UTMDACC treated between 2015–2020, revealed significant differences in OS and RFI driven by clinical and treatment differences.

Although RT was predominant in both cohorts, significantly more patients received RT alone in the Copenhagen cohort, which is noteworthy, since the Copenhagen cohort presented at higher UICC-stage, T- and N-class. In line with this, RT remains the predominant treatment modality in most European centers [19].

The UTMDACC cohort included more clinical characteristics associated with a better prognosis: more HPV+ OPSCC patients, lower T- and N-burden and less smoking [20] but also more men which are associated with a worse prognosis [21]. When adjusting for these differences, an independent increased OS HR was not observed, but the risk of recurrence was significantly higher in the Copenhagen cohort (RFI HR 1.74, $p = 0.003$). Discrepancies in follow-up regimens and divergent definitions of recurrence may also attribute to the observed disparities. Although speculative, it could also indicate a different biology of HPV+ OPSCCs. In line with our findings, a recent study including a Danish and a Toronto cohort observed significant clinical and treatment differences, which were reflected in differences in locoregional failure and OS [22].

In low-risk patients, significant clinical and treatment differences were observed with 41.7 % in the Copenhagen cohort receiving RT alone versus 19.6 % in the UTMDACC cohort, yet the prognosis remained equally good. Several studies have investigated de-escalated treatment to minimize treatment-related toxicities for a subgroup of patients with HPV+ OPSCC [8,9,23–25]. It is important to keep in mind that substantial clinical differences exist when selecting patients and validating de-escalating trials in various geographical areas. This study underlines the importance of including multiple factors like TNM-classification, HPV- and smoking status, but also indicates that factors like age and tumor location could be considered for stratification.

Conversely, a sub-analysis of high-risk patients revealed significant clinical and treatment differences influencing the prognosis. The UTMDACC cohort had significantly better OS and RFI. OS was also significantly associated with current smoking, stage, and treatment modality given. RT alone was given to a substantial part of the Copenhagen patients (37.1 % vs 7.2 %), despite that CRT is recommended for this group [16,17]. The difference in use of concurrent chemotherapy is likely a result of factors impacting treatment selection not included in this study, e.g., comorbidities, as the Copenhagen cohort is older and include more smokers. But a difference in practice patterns cannot be excluded. Neoadjuvant chemotherapy is not used in Denmark but was given to 16.7 % of the patients in the UTMDACC cohort (corresponding to 39.1 % of UTMDACC high-risk patients), which is in line with data from The National Cancer Database [26]. The role of neoadjuvant therapy in locally advanced OPSCC cancer remains controversial [27], however, it may be associated with a decreased risk of distant metastasis [28]. A recent study suggests that neoadjuvant therapy is associated with an improved OS and lower risk of distant metastasis in patients with OPSCC [29].

A notable difference in treatment between MDA and Denmark is the use of hypoxic modification with nimorazole. The DAHANCA 5 randomized trial, investigated the effect of nimorazole, showed a significantly better loco-regional control rate and lower cancer-related death in patients receiving nimorazole compared to placebo [30]. Nimorazole has since been standard of care for all Danish OPSCC patients. The benefit of nimorazole may primarily be present in HPV- OPSCC, as reanalysis showed no significant benefit in HPV/p16-positive tumors [31].

Disparities in the healthcare system might introduce a selection bias contributing to the differences observed in this study, with more HPV- OPSCC patients and more high-risk patients in the Copenhagen cohort.

Table 3
Multi- and univariable analysis for recurrence-free interval for OPSCC in Eastern Denmark and UTMDACC from 2015–2020.

	Univariable			Multivariable[e]		
Variable	HR	95 % CI	p-value[d]	HR	95 % CI	p-value[d]
Center (UTMDACC ref)						
Eastern Denmark	2.11	1.63-2.74	< 0.001[d]	1.74	1.21-2.50	0.003[d]
Age (<60 years ref)						
> 60 years	1.53	1.20-1.96	< 0.001[d]	1.08	0.83-1.42	0.56
Gender (female ref)						
Male	1.01	0.75-1.38	0.93	1.19	0.86-1.64	0.30
HPV-status (HPV+ ref)						
HPV-	3.30	2.58-4.24	< 0.001[d]	2.10	1.50-2.96	< 0.001[d]
Smoking status (never ref)						
previous	1.55	1.13-2.12	0.01[d]	1.11	0.80-1.55	0.53
current	3.12	2.27-4.28	< 0.001[d]	1.31	0.88-1.95	0.19
Tumor location (tonsil ref)						
BOT	1.04	0.79-1.37	0.76	0.96	0.72-1.28	0.78
Other	2.44	1.77-3.36	< 0.001[d]	1.12	0.78-1.63	0.53
T-class (T1 ref)						
T2	1.73	1.20-2.48	0.003[d]	1.71	1.17-2.49	0.005[d]
T3	3.11	2.12-4.58	< 0.001[d]	2.68	1.75-4.09	< 0.001[d]
T4	4.16	2.86-6.04	< 0.001[d]	3.12	2.05-4.75	< 0.001[d]
N-class (N0 ref)						
N1	0.73	0.52-1.04	0.08	1.67	1.13-2.47	0.01[d]
N2	2.07	1.45-2.96	< 0.001[d]	2.49	1.66-3.72	< 0.001[d]
N3	3.17	1.85-5.43	< 0.001[d]	4.07	2.27-7.32	< 0.001[d]
M-class (M0 ref)				3.22	1.46-7.10	0.004[d]
M1	3.49	1.65-7.41	0.001[d]	3.22	1.46-7.10	0.004[d]
Treatment regimen (CRT ref)						
RT	2.19	1.66-2.89	< 0.001[d]	1.98	1.45-2.70	< 0.001[d]
Neoadjuvant chemotherapy + RT/CRT	1.86	1.24-2.77	0.002[d]	2.18	1.32-3.60	0.002[d]
Surgery	0.90	0.58-1.40	0.64	1.64	0.99-2.71	0.05
Surgery + RT/CRT	0.60	0.30-1.18	0.14	0.96	0.46-2.01	0.91

Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; ref, reference; HPV, human papillomavirus; BOT, base of tongue; RT, radiotherapy; CRT, chemoradiotherapy.

[d] Significant value.

[e] Adjusted for center, age group, gender, HPV status, smoking status, T-site location, stage UICC8 and treatment regimen.

Studies have shown that insurance status is predictive of clinical characteristics such as tumor stage and comorbidities at diagnosis as well as oncological outcome [32–34]. A recent Copenhagen study comprising a part of the Copenhagen cohort suggests that low socioeconomic status negatively impact the OS, largely due to differences in clinical characteristics at diagnosis, including smoking status, comorbidities, and clinical stage [35]. Future studies including socioeconomic status, comorbidities, and performance status would provide further insight into this matter. Disparities in the healthcare system might also contribute to the differences observed in treatment modalities, availability, and regimen of follow-up care.

4.1. Limitations

A recent study, where the Copenhagen cohort comprised the majority of the study cohort, has proven double HPV/p16-positivity to be superior in prognostication and has led to the recommendation of including both p16 and HPV-status in areas with high discordance, which is also supported by the ESMO guidelines [4,5]. In this study, HPV-positivity was defined in the UTMDACC as p16 + or HPV+ , which might overestimate the HPV-positive prevalence, although only 1.9 % had discordant p16/HPV status [4]. The included cohorts might not be representative of the Northern American and European cohorts overall, and the results may not apply to other regions. Socioeconomic status, performance score, and comorbidities were not included which potentially impacts treatment decisions and have shown to influence both OS and RFI and which we hypothesize differs greatly in the two cohorts.

5. Conclusion

Significant demographic, clinical, and treatment differences influencing the prognosis were identified between two high HPV-prevalence OPSCC cohorts representing two different health care systems: a Copenhagen cohort from a public, universal health care system and a UTMDACC cohort from an insurance-based health care system. Despite significant clinical and treatment differences, the prognosis for low-risk OPSCC patients was equally good, while the prognosis was significantly better for patients with high-risk OPSCC in the UTMDACC cohort.

Our study suggests that significant clinical differences exist between a Northern European and Northern American OPSCC population, which is important to consider when comparing outcomes and for patient stratification in clinical trials, as reproducibility might be challenging. However, to fully understand possible intercontinental variations, more data are needed.

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The funder had no role in study design, in the collection, analysis or interpretation of data, in the writing of the report or in the decision to submit this article for publication.

Declaration of Interest statement

None declared.

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CRediT authorship contribution statement

Christian Grønhøj: Conceptualization, Writing – review & editing. **Martin Garset-Zamani:** Data curation, Investigation, Writing – review & editing. **Faye M. Johnson:** Writing – review & editing. **Katherine Hutcheson:** Writing – review & editing. **Amy C. Moreno:** Conceptualization, Resources, Supervision, Writing – review & editing. **Clifton D. Fuller:** Project administration, Writing – review & editing. **Neil D. Gross:** Writing – review & editing. **Simone Kloch Bendtsen:** Project administration, Writing – review & editing. **Toyin Babarinde:** Conceptualization, Writing – review & editing. **Amanda-Louise F. Carlander:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization,

Writing – original draft, Writing – review & editing. **Christian von Buchwald:** Conceptualization, Resources, Supervision, Writing – review & editing. **Kathrine Kronberg Jakobsen:** Methodology, Writing – review & editing. **Jeffrey N. Myers:** Conceptualization, Resources, Supervision, Writing – review & editing. **Jacob Høyer Rasmussen:** Conceptualization, Methodology, Writing – review & editing. **Jeppe Friborg:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2024.113983.

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Supplement 1

1. Methods and Materials

Variables

The last day of follow-up for the Copenhagen cohort was defined as the last clinical visit, death, or recurrence; and for the UTMDACC cohort as the last imaging, death, or recurrence. Recurrence was defined as local, regional and/or distant. Head and neck cancer within the first 6 months after primary OPSCC was considered progression, i.e., not a recurrence in both cohorts. For the Copenhagen cohort, a new diagnosis of OPSCC identified from six months to five years after primary diagnosis and treatment was considered a recurrence and not a second primary unless specifically stated otherwise in the medical record. For the UTMDACC cohort, the distinction between recurrence or second primary was determined by the treating team based on proximity to the initial cancer and time from treatment (<5 years).

Measurement data

Age was grouped as <60 years and >60 years. Patient-reported smoking status at the time of diagnosis was grouped as never smoking/former smoking/current smoking. HPV-positivity was defined as being positive to both p16 immunohistochemistry and HPV DNA in the Copenhagen cohort, while HPV-positivity was defined as being positive to either p16 or HPV DNA/RNA. Clinical stage was defined according to the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC) TNM classification system (8^{th} edition). For p16- tumors, stages IVA-C were merged to IV. Treatment regimen as assigned at a multidisciplinary treatment planning conference and grouped as radiotherapy (RT)/chemoradiotherapy (CRT)/neoadjuvant chemotherapy + RT/CRT/surgery/surgery +RT/CRT. Tumor location was grouped as palatine tonsils/base of tongue/other OPSCC location. Other oropharyngeal sites included in the Copenhagen cohort were pharyngeal wall, soft palate, uvula or pharyngeal arch, while sites included in the UTMDACC cohort were soft palate, pharyngeal wall and glossopharyngeal sulcus.

HPV detection and p16 immunohistochemistry (IHC)

For the Copenhagen cohort, HPV DNA was detected with PCR as previously described from 2015-2017[13-15]. From 2018-2020 the presence of HPV DNA and HPV genotypes was evaluated by VisionArray HPV chip 1.0 (ZytoVision). p16 IHC was carried out using Ventana Benchmark Ultra auto-stainer with the UltraView detection kit and the p16 monoclonal antibody E6H4 ready-to-use with CC1 as pretreatment (Roche,

Tuscon, USA). The p16-positivity cutoff was set at >70% for both nuclear and cytoplasmatic staining. HPV genotypes was detected with next generation sequencing from 2000-2017 as previously described[13-15].

For the Stiefel Database, the presence of HPV virus was detected by HPV DNA in situ hybridization, HPV RNA in situ hybridization, cytology genotyping, or HPV DNA PCR. The p16-positivity was assessed by IHC.

Treatment

In Denmark, curative radiation-based therapy is the primary treatment and consists of moderately accelerated radiotherapy (RT) given in 33-34 fractions 6 days a week with or without concurrent chemotherapy (CRT). The standard dose levels are 66-68 Gray (Gy) to the clinical target volume (CTV) 1, and 60 Gy to the CTV2. Dose level to elective targets is 50 Gy. According to guidelines, CRT is given based on an evaluation of performance status and comorbidities and consists mainly of weekly cisplatin (40mg/m^2). Carboplatin is given if cisplatin is not tolerated. Very few patients received cetuximab. Induction therapy is not used in Denmark. Trans-oral robotic surgery (TORS) is considered for patients in clinical trials or if primary RT cannot be performed. In Denmark, all patients were also offered concurrent nimorazole, a radiosensitizer, unless there were contraindications. Unilateral RT could be offered for well-lateralized tonsil carcinomas without involvement of midline structures[16,17].

At UTMDACC, radiation therapy for OPSCC is generally delivered alone or with concurrent systemic therapy over the course of 6 to 7 weeks (i.e., 33-34 fractions) to a curative total dose of 60-70 Gy. The standard dose levels include 66-70 Gy to CTV1 (i.e., gross tumor with anatomically restricted expansion), 60-63 Gy to adjacent at-risk primary sites, involved neck levels or postoperative volumes, and 54-57 Gy to elective clinical targets (i.e., elective nodal irradiation volumes). Unilateral RT is reserved for well-lateralized clinical T1-T2 tonsil carcinomas with no tongue base involvement, <1cm soft palate involvement, and minimal adenopathy. Concurrent chemotherapy is primarily given as weekly cisplatin (40 mg/m^2), which can be switched to carboplatin if cisplatin is not tolerated. Currently, few patients who are ineligible to cisplatin receive cetuximab. TORS is considered for all patients with unilateral, low-volume disease (T0-2, N0-1) OPSCC[18].

Statistics

Subgroup analyses were performed for both low-risk patients (T1/T2N0M0) and high-risk patients (stage III-IV). For the T1T2N0M0 analyses the multivariable OS analysis was adjusted for center, HPV-status, smoking

status, T-site location, and stage UICC8 based on clinical relevance and due a limited number of events (50). Likewise, the multivariable RFI analysis was adjusted for center, HPV status and stage UICC8 based on clinical relevance and due a limited number of events (26). For the III-IV analyses, the multivariable OS analysis (192 events) was adjusted for center, HPV-status, smoking status, stage UICC8 and treatment regimen while the multivariable RFI (126 events) analysis was adjusted for center, HPV-status, stage UICC8, treatment regimen based on clinical relevance.

2. Results

Subgroup analysis of patients receiving RT as single modality

The Copenhagen RT single modality cohort comprised significantly more men (27.2% vs 12.2%, p<0.001), more HPV- OPSCC (47.6% vs 9.0%, p<0.001), more other OPSCC location (23.1% vs 5.9%, p<0.001), older age (mean age 68.4 vs 61.3 years, p<0.001), more current smokers (45.1% vs 7.5%, p<0.001) and more stage III-IV (35.9% vs 8.3%, p<0.001). 3-year OS in the Copenhagen RT single modality cohort was 61.6% (95% CI: 56.2-67.5%) and in the UTMDACC RT single modality cohort 88.6% (95% CI: 83.4-94.1%), log-rank p=0.001. Data not shown.

Table 2. 1- and 3-year overall survival estimates stratified by center and HPV-status from 2015-2020.

Overall survival 2015-2020	**Eastern Denmark**	**UTMDACC, USA**
OS total	% (95% CI)	% (95% CI)
1-year	92% (91-94%)	96% (95-97%)
3-year	83% (80-85%)	91% (89-93%)
OS HPV+		
1-year	96% (94-97%)	97% (96-98%)
3-year	88% (86-91%)	93% (91-95%)
OS HPV-		
1-year	85% (81-88%)	96% (92-100%)
3-year	56% (51-62%)	78% (68-89%)

Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; OS, overall survival; HPV, Human Papillomavirus.

Table 3. Characteristics of 299 patients with T1/T2N0M0 OPSCC in Eastern Denmark and UTMDACC, Texas, USA from 2015-2020.

Variable	Eastern Denmark, n=192		UTMDACC, Texas USA, n=107		
	no.	%	no.	%	*p-value**
Gender					*<0.001**
Male	126	65.6	80	74.8	
Female	66	34.4	27	25.2	
Median age (IQR)	65 (59-72)		61 (55-67)		*<0.001**
no.	192		107		
Median follow-up. years (IQR)	2.2 (1.4-3.2)		1.9 (0.8-3.5)		0.34
no.	192		107		
Smoking					*<0.001**
Current	108	56.3	5	4.7	
Former	61	31.8	40	37.4	
Never	22	11.5	60	56.1	
Unknown	1	0.5	2	1.9	
HPV-status					*<0.001**
HPV+	69	35.9	91	85.0	
HPV-	121	63.0	14	13.1	
Unknown	2	1.0	2	1.9	
Tumor location					*<0.001**
BOT	24	12.5	33	30.8	
Tonsils	84	43.8	64	59.8	
Other	84	43.8	10	9.3	
TNM Stage (UICC/AJCC 8), HPV+					
T-class					1
T1	20	29.0	27	29.7	
T2	49	71.0	64	70.3	
TNM Stage (UICC/AJCC 8), HPV-					
T-class					0.68
I	55	45.5	5	35.7	
II	66	54.5	9	64.3	
Overall stage (UICC/AJCC8), HPV-					0.48
I	60	49.6	5	35.7	
II	61	50.4	9	64.3	
Treatment					*<0.001**
CRT	9	4.7	25	23.4	
RT	80	41.7	21	19.6	
Neoadjuvant chemotherapy + RT/CRT	-	-	1	0.9	
Surgery	101	52.6	45	42.1	

Surgery + RT/CRT	2	1	15	14

Note: Frequency (%) is provided for categorical variables, median (IQR) are provided for continuous variables. Chi Square test were used for categorical variables, while t-test was used for continuous variables. Fisher's exact test was used for small sample sizes.
Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; IQR, interquartile range; HPV, Human Papillomavirus; BOT, base of tongue; RT, radiotherapy; CRT, concurrent RT + systemic therapy.
*Significant p-value

Table 4. Multi- and univariable analysis for overall survival for T1/T2N0M0 OPSCC in Eastern Denmark and UTMDACC from 2015-2020.

	Univariable			Multivariable**		
Variable	**HR**	**95% CI**	**p-value***	**HR**	**95% CI**	**p-value***
Center (UTMDACC ref)						
Eastern Denmark	2.63	1.23-5.62	0.01*	0.94	0.36-2.42	0.90
Age (<60 years ref)				-		
>60 years	1.83	0.55-0.99	0.06			
Gender (female ref)				-		
Male	1.19	0.64-2.21	0.58			
HPV-status (HPV+ ref)						
HPV-	3.96	2.11-7.40	<0.001*	1.92	0.70-5.21	0.20
Smoking status (never ref)						
Previous	0.96	0.36-2.58	0.93	0.74	0.26-2.11	0.58
Current	3.97	1.75-9.01	<0.001*	2.11	0.71-6.27	0.18
Tumor location (tonsils ref)						
BOT	0.81	0.30-2.19	0.67	0.72	0.26-2.0	0.53
Other	2.99	0.163-5.49	<0.001*	1.40	0.65-3.0	0.39
UICC stage (I ref)						
UICC II	2.56	1.45-4.49	0.001*	0.98	0.49-1.94	0.94
Treatment (CRT ref)				-		
RT	1.73	0.66-4.54	0.26			
Surgery	0.78	0.29-2.1	0.62			
Surgery + RT/CRT	0.85	0.16-4.36	0.84			

Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; ref, reference; HPV, Human Papillomavirus; BOT, base of tongue; RT, radiotherapy; CRT, concurrent RT + systemic therapy.
*Significant p-value. **Adjusted for center, HPV-status, smoking status, tumor location and stage UICC8.

Table 5. Multi- and univariable analysis for recurrence-free interval for T1/T2N0M0 OPSCC in Eastern Denmark and UTMDACC from 2015-2020.

	Univariable			Multivariable**		
Variable	**HR**	**95% CI**	**p-value***	**HR**	**95% CI**	**p-value***
Center (UTMDACC ref)						
Eastern Denmark	5.32	1.25-22.6	0.02*	3.14	0.67-14.61	0.15
Age (<60 years ref)				-		
>60 years	2.21	0.88-5.53	0.09			
Gender (female ref)				-		
Male	1.30	0.54-3.12	0.56			
HPV-status (HPV+ ref)						
HPV-	4.10	1.63-10.33	0.002*	2.08	0.66-6.60	0.21
Smoking status (never ref)				-		
Previous	1.08	0.65-14.51	0.15			
Current	5.51	1.26-24.17	0.02*			
Tumor location (tonsils ref)				-		
BOT	1.50	0.44-5.13	0.52			
Other	3.27	1.31-8.13	0.01*			
UICC stage (I ref)				1.73	0.68-4.39	0.25
UICC II	3.19	1.45-7.0	0.004*			

Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; ref, reference; HPV, Human Papillomavirus; BOT, base of tongue. *Significant p-value.** Adjusted for HPV-status, center and stage UICC8.

Table 6. Characteristics of 560 patients with OPSCC stage III-IV in Eastern Denmark and UTMDACC, Texas, USA from 2015-2020.

Variable	Eastern Denmark, n=353		UTMDACC, Texas USA, n=207		
	no.	**%**	**no.**	**%**	*p-value**
Gender					*<0.001**
Male	260	73.7	190	91.8	
Female	93	26.3	17	8.2	
Median age (IQR)	64 (58-70)		63 (57-68)		*0.02**
no.	353		207		
Median follow-up. years (IQR)	1.9 (1.2-2.8)		2.1 (1.1-3.6)		*0.02**
no.	192		207		
Smoking					*<0.001**
Current	221	62.6	19	9.2	
Former	104	29.5	106	51.2	
Never	28	7.9	81	39.1	
Unkown	0		1	0.5	
HPV-status					*<0.001**
HPV+	82	23.2	139	67.1	
HPV-	270	76.5	68	32.9	
Unknown	1	0.3	0	-	
Tumor location					*<0.001**
BOT	137	38.8	127	61.4	
Tonsils	114	32.3	66	31.9	
Other	102	28.9	14	6.8	
TNM Stage (UICC/AJCC 8), HPV+					
T-class					0.86
T1	8	9.8	10	7.2	
T2	9	11.0	13	9.4	
T3	30	36.6	56	40.3	
T4	35	42.7	60	43.2	
N-class					*0.002**
N0	0	-	1	0.7	
N1	0	-	4	2.9	
N2	53	64.6	112	80.6	
N3	29	35.4	22	15.8	
M-class					*0.003**
M0	79	96.3	125	89.9	
M1	1	1.2	14	10.1	

Unkown		2	2.4	0	-	
Overall stage (UICC/AJCC8), HPV+						*0.007**
III		81	98.8	124	89.2	
IV		1	1.2	15	10.8	
TNM Stage (UICC/AJCC 8), HPV-						
T-class						0.57
T1	45	16.7	11	16.2		
T2	63	23.3	22	32.4		
T3	83	30.7	19	27.9		
T4	79	29.3	16	5.9		
N-class						0.44
N0	37	13.7	6	8.8		
N1	54	20	19	27.9		
N2	155	57.4	37	54.4		
N3	24	8.9	6	8.8		
M-class						0.23
M0		264	97.8	66	97.1	
M1		6	2.2	1	1.5	
Unknown		0	-	1	1.5	
Overall stage (UICC/AJCC8), HPV-						1
III		72	26.7	18	26.5	
IV		198	73.3	50	73.5	
Treatment						*<0.001**
CRT		201	56.9	103	49.8	
RT		132	37.4	15	7.2	
Neoadjuvant chemotherapy + RT/CRT				81	39.1	
Surgery		12	3.4	1	0.5	
Surgery RT/CRT		8	2.3	7	3.4	

Note: Frequency (%) is provided for categorical variables, median (IQR) are provided for continuous variables. Chi Square test were used for categorical variables, while t-test was used for continuous variables. Fisher's exact test was used for small sample sizes.
Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; IQR, interquartile range; HPV, Human Papillomavirus; BOT, base of tongue; RT, radiotherapy; CRT, concurrent RT + systemic therapy.
*Significant p value

Table 7. Multi- and univariable analysis for overall survival for OPSCC UICC8 Stage III/IV in Eastern Denmark and UTMDACC from 2015-2020.

	Univariable			Multivariable**		
Variable	**HR**	**95% CI**	**p-value***	**HR**	**95% CI**	**p-value***
Center (UTMDACC ref)						
Eastern Denmark	2.51	1.76-3.57	<0.001*	2.20	1.29-3.75	0.004*
Age (<60 years ref)				-		
<60 years	1.07	0.79-1.43	0.68			
Gender (female ref)				-		
Male	0.93	0.66-1.31	0.67			
HPV-status (HPV+ ref)						
HPV-	1.91	1.39-2.63	<0.001*	0.76	0.46-1.26	0.29
Smoking status (never ref)						
Previous	1.88	1.14-3.12	0.01*	1.52	0.89-2.58	0.23
Current	3.00	1.85-4.85	<0.001*	1.85	1.03-3.30	0.04*
Tumor location (tonsils ref)				-		
BOT	0.74	0.53-1.03	0.07			
Other	1.06	0.74-1.52	0.74			
UICC stage (III ref)						
UICC IV	1.85	1.39-2.48	<0.001*	1.80	1.24-2.62	0.002*
Treatment (CRT ref)						
RT	2.34	1.72-3.19	<0.001*	2.20	1.59-3.04	<0.001*
Neoadjuvant chemotherapy + RT/CRT	0.84	0.52-1.35	0.47	1.84	0.96-3.51	0.07
Surgery	0.83	0.30-2.32	0.72	0.75	0.26-2.14	0.59
Surgery + RT/CRT	0.86	0.35-2.11	0.74	1.07	0.42-2.72	0.88

Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; ref, reference; HPV, Human Papillomavirus; BOT, base of tongue; RT, radiotherapy; CRT, concurrent RT + systemic therapy. *significant p-value. **Adjusted for center, HPV-status, smoking status and treatment regimen.

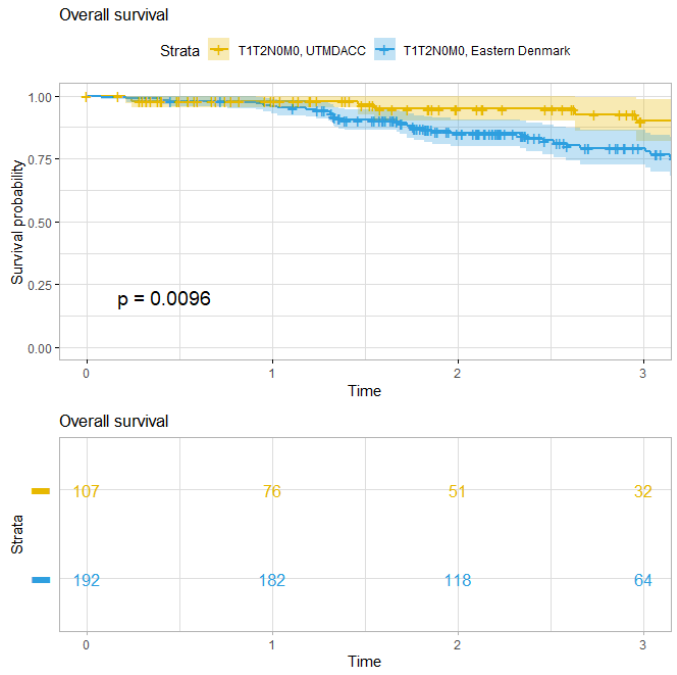
Table 8. Multi- and univariable analysis for recurrence-free interval for OPSCC UICC8 Stage III/IV in Eastern Denmark and UTMDACC from 2015-2020.

	Univariable			Multivariable**		
Variable	**HR**	**95% CI**	**p-value***	**HR**	**95% CI**	**p-value***
Center (UTMDACC ref)						
Eastern Denmark	1.95	1.30-2.93	0.001*	2.80	1.46-5.38	0.002*
Age (<60 years ref)				-		
>60 years	0.97	0.67-1.38	0.85			
Gender (female ref)				-		
Male	0.95	0.62-1.46	0.81			
HPV-status (HPV+ ref)						
HPV-	1.52	1.04-2.22	0.03*	0.74	0.41-1.33	0.32
Smoking status (never ref)				-		
Previous	1.11	0.67-1.88	0.67			
Current	1.48	0.90-2.44	0.12			
Tumor location (tonsils ref)				-		
BOT	0.92	0.61-1.40	0.70			
Other	1.46	0.94-2.270	0.09			
UICC stage (III ref)						
UICC IV	1.92	1.34-2.74	<0.001*	2.20	1.35-3.57	0.001*
Treatment (CRT ref)						
RT	1.90	1.28-2.81	0.001*	1.84	1.22-2.76	0.004*
Neoadjuvant chemotherapy + RT/CRT	1.29	0.77-2.15	0.33	3.10	1.45-6.65	0.004*
Surgery	0.66	0.16-2.71	0.57	0.72	0.17-2.98	0.65
Surgery + RT/CRT	0.81	0.25-2.60	0.73	1.13	0.34-3.70	0.85

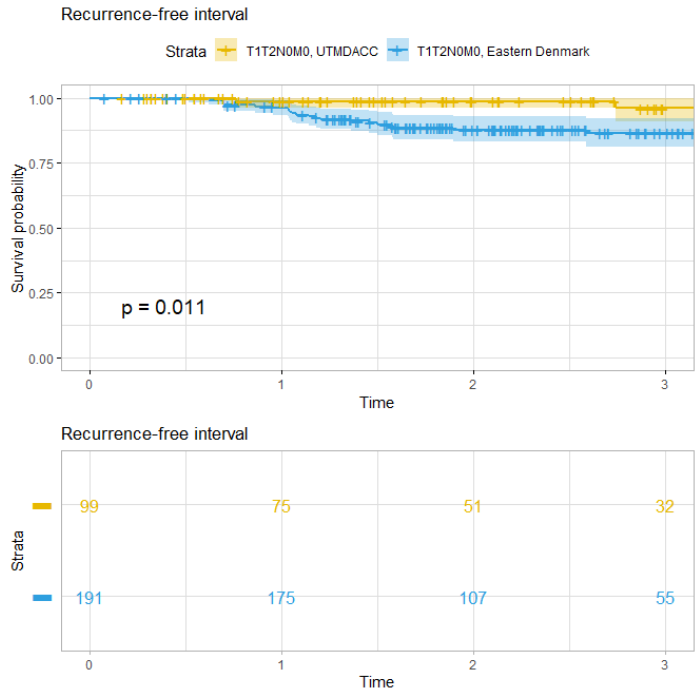
Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA; ref, reference; HPV, Human Papillomavirus; BOT, base of tongue; RT, radiotherapy; CRT, concurrent RT + systemic therapy. *Significant p-value. ** Adjusted for center, HPV-status, stage UICC8, treatment regimen.

Figure 1. Kaplan-Meier curves depicting the overall survival and recurrence-free interval stratified by center for T1/T2N0M0 OPSCC from 2015-2020. A. Overall survival. B. Recurrence-free interval.

A.



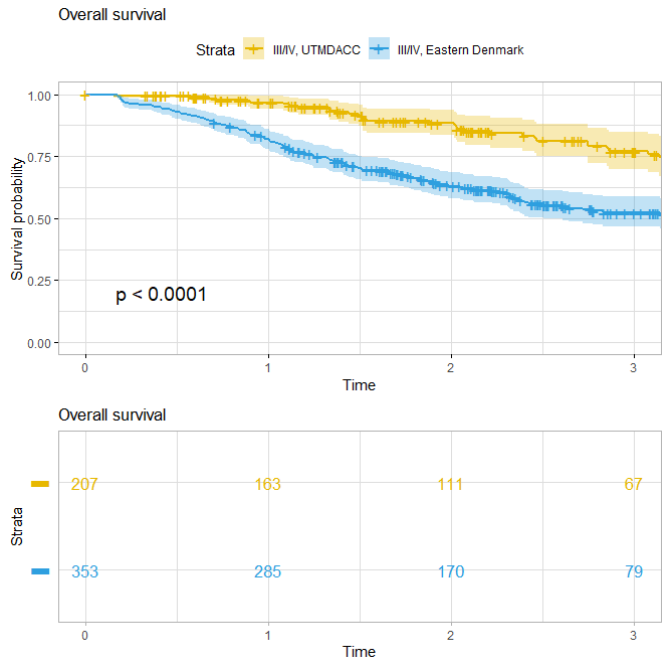
B.



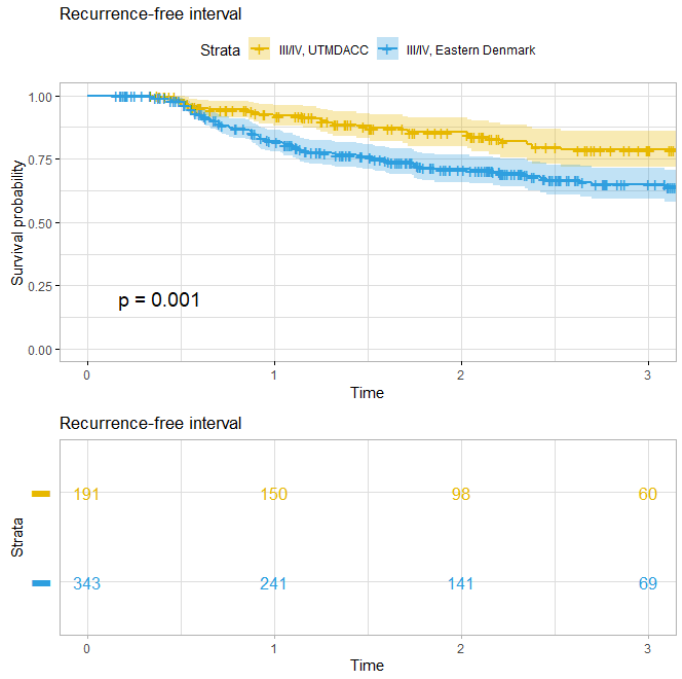
Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA, HPV, Human Papillomavirus; OPSCC, oropharyngeal squamous cell carcinoma.

Figure 2. Kaplan-Meier curves depicting the overall and recurrence-free interval stratified by center for OPSCC UICC8 stage III-IV from 2015-2020. A. Overall survival. B. Recurrence-free interval.

A.



B.



Abbreviations: UTMDACC, The University of Texas MD Anderson Cancer Center, USA, HPV, Human Papillomavirus; OPSCC, oropharyngeal squamous cell carcinoma.

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Mesenchymal Stromal/Stem Cell Therapy Improves Salivary Flow Rate in Radiation-Induced Salivary Gland Hypofunction in Preclinical in vivo Models: A Systematic Review and Meta-Analysis

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Abstract

Background Mesenchymal stromal/stem cells (MSCs) have been suggested for salivary gland (SG) restoration following radio-induced salivary gland damage. This study aimed to determine the safety and effectiveness of MSC therapy on radio-induced SG damage and hypofunction in preclinical in vivo studies.

Methods PubMed and EMBASE were systematically searched for preclinical in vivo interventional studies evaluating efficacy and safety of MSC treatment following radio-induced salivary gland damage published before 10th of January 2022. The primary endpoint was salivary flow rate (SFR) evaluated in a meta-analysis. The study protocol was published and registered on PROSPERO (www.crd.ac.uk/prospero), registration number CRD42021227336.

Results A total of 16 preclinical in vivo studies were included for qualitative analysis (858 experimental animals) and 13 in the meta-analysis (404 experimental animals). MSCs originated from bone marrow (four studies), adipose tissue (10 studies) and salivary gland tissue (two studies) and were administered intravenously (three studies), intra-glandularly (11 studies) or subcutaneously (one study). No serious adverse events were reported. The overall effect on SFR was significantly increased with a standardized mean difference (SMD) of 6.99 (95% CI: 2.55–11.42). Studies reported improvements in acinar tissue, vascular areas and paracrine factors.

Conclusion In conclusion, this systematic review and meta-analysis showed a significant effect of MSC therapy for restoring SG functioning and regenerating SG tissue following radiotherapy in preclinical in vivo studies without serious adverse events. MSC therapy holds significant therapeutic potential in the treatment of radio-induced xerostomia, but comprehensive, randomized, clinical trials in humans are required to ascertain their efficacy in a clinical setting.

Keywords Xerostomia · Radiotherapy · Cell Therapy · Mesenchymal stem Cells · Systematic Review

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Introduction

Most patients with head and neck cancer (HNC) are treated with radiotherapy [1]. Salivary gland (SG) hypofunction and xerostomia, the subjective feeling of dry mouth, are common and long-term side effects following radiotherapy in the head and neck area [2]. Despite the emergence of intensity-modulated radiation-therapy (IMRT), that to some extent spare the surrounding tissue due to a more precise delivery to target tissue, the SG are often damaged [3, 4]. Radio-induced SG damage is dose-dependent and leads to gland degeneration and progressive decline in saliva production, followed by complications such as xerostomia, problems with speech and swallowing, oral infections and dental caries thus reducing quality of life. Currently, only symptomatic treatments are available, and there is a lack of regenerative and restorative therapeutic options [2, 5–7].

Mesenchymal stromal/stem cells (MSCs) are multipotent adult progenitor cells that in vitro can differentiate into mesodermal lineages with abilities for tissue regeneration and which can be isolated from numerous connective tissues, e.g. bone marrow (MSCs[M]) and adipose tissue (MSCs[AT]) [8–10]. Aside from being easily accessible, MSCs encompass various advantages such as proliferative and differentiating capacities; but also, immunomodulatory, and trophic properties such as anti-inflammatory, anti-fibrotic, anti-apoptotic, angiogenetic and immunosuppressive effects [11–13]. Thus, MSCs have a therapeutic and disease-modifying potential to repair and/or restore radio-induced SG damage. Recent preclinical in vivo studies have focused on mesenchymal stem cell (MSC) transplantation to repair radiation damaged SGs as a potentially curative treatment for SG hypofunction [14, 15]. Also, MSC therapy have shown to improve salivary flow rate (SFR) in humans [16–19].

Nevertheless, while MSC therapy shows potential as a treatment option for radio-induced SG damage, existing studies have been limited in size, characterized by high heterogeneity in relation to MSC origin, and only a few have been conducted in both preclinical in vivo models and humans. The use of MSCs therapy for radio-induced SG hypofunction alone has not yet been evaluated in a systematic review and meta-analysis. The aim of this study was therefore to review the safety and effectiveness of MSC therapy for restoring SG function after radiation-induced damage in preclinical in vivo studies. This is of great importance to optimize clinical trials and to assess the prospective implication in the curative treatment of SG hypofunction caused by radiotherapy.

Method

The study adheres to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocols (PRISMA-P) statement and the study protocol was published and registered on PROSPERO (www.crd.ac.uk/prospero), registration number CRD42021227336 [20].

We considered preclinical in vivo models that assessed MSC therapy following experimentally induced radiation injury of major SGs. Inclusion criteria were: (1) preclinical in vivo intervention studies of both sexes and all ages (2) exposure of SGs to ionizing radiation (3) MSC therapy of all administration routes. There were no restrictions regarding induction of radiation damage, but MSCs administration should be after induction of radiation injury. MSC secretome, exosomes, and treatment with parts of MSCs were also included. In vitro models, treatments other than MSCs, SG damage other than ionizing radiation and non-relevant outcome were excluded. Human studies were not included in the formal analysis but described and included the discussion. The primary outcome was efficacy measured by SFR and secondary outcomes was SG morphology, SG histology, changes in saliva composition, circulating immune cells, SG paracrine effects, mode of action and safety in terms of objective adverse events as previously described [20].

Systematic Search

In January 2022 two authors (ALFC, CH) systematically searched PubMed and Embase for preclinical in vivo interventional studies assessing the efficacy and safety of MSC therapy for radiation-induced SG hypofunction. The search was performed using Medical Subject Headings (MeSH), Emtrees and text words relating to MSCs, SG hypofunction, SG damage, SG dysfunction, radiation-induced SG damage or xerostomia. The specific search string for PubMed and Embase was previously described [20]. The search of databases reference lists was evaluated for additional relevant studies.

Data Extraction

Two authors (ALFC, CH) independently screened all articles for eligibility and disagreement was solved by consensus or by discussion with a third reviewer (BM or CDL). The following information was extracted from each study: (1) article information (author, publication year), (2) details on preclinical in vivo model (species, sex, sample size, age), (3) study design (controlled, uncontrolled, randomized and/or blinded), (4) irradiation details (dose, Gy, quality assurance and days from irradiation to MSC therapy), 6) MSC therapy (type, concentration, administration route,

follow-up time), 7) statistical analysis, 8) outcomes (functional and molecular outcomes).

Quality Assessment and Risk of Bias

We assessed the quality of reporting in the included studies according to the latest Animal Research: reporting of in vivo Experiments (ARRIVE) guidelines [21]. One point was given for evidence of each quality criterion. The methodological quality was assessed using the SYRCLE (Systematic review Center for Laboratory Animal Experimentation) risk of bias tool in domains related to selection bias, performance bias, detection bias, attrition bias and reporting bias [22].

Data Analysis

A descriptive summary of all outcomes was performed. The efficacy of MSC therapy was evaluated by a random effect meta-analysis adjusted to Hedge's g on SFR. If there were multiple time points, only the last one was included in the meta-analysis. A standardized mean difference (SMD) with 95% confidence intervals (CIs) was used to evaluate the effect on SFR. The SMD was calculated by dividing the difference in mean outcome between the groups with the standard deviation of outcome among participants as per recommendation by the Cochrane Handbook [23]. Heterogeneity of the study results was investigated using the Cochrane Q test and quantified with I^2 values. Subgroup analyses were performed for species, strain, sex, administration route, age in weeks, radiation duration, frequency of treatment, radiation dose and time between radiation to MSC treatment. Sub analysis on frequency of radiation was not performed since radiation was administered as a single treatment in all studies.

Results

Sixteen preclinical studies published between 2011 and 2019 met the inclusion criteria, see Fig. 1 [24–39]. One human study [19] ($n=30$, intervention group $n=15$) were identified. All preclinical in vivo studies were included in the quantitative (858 experimental animals) and 13 (404 experimental animals) were also included in the qualitative analyses, see Fig. 1.

Description of the Preclinical in vivo Models

All studies investigated the safety and efficacy of MSC-based therapy for xerostomia and SG hypofunction following radiation in the head and neck area. A total of 858 animals were included of which 341 received intervention with MSC therapy. Ten studies included mice, two studies rats and one study miniature pigs. The irradiation dose varied from 10 to 25 Gy, and all were administered as a single dose. See Table 1.

MSCs were originating from bone marrow (4 studies) [24, 27, 36, 37], adipose tissue (10 studies) [25, 28–33, 35, 38, 39], and salivary gland tissue (2 studies) [26, 34] and were administered intravenously (3 studies) [28, 31, 39], intra-glandularly (11 studies) [24–27, 29, 32–36, 38] or subcutaneously (1 study) [30]. One study did not specify administration route [37]. One study used secretomes originating from adipose tissue [39]. The mean follow-up time was 12 weeks after MSC treatment, ranging from 7 days to 24 weeks. See Table 1.

Safety of MSC

All included preclinical in vivo studies described the MSC-based cell therapy as safe with no reported serious adverse events [24–39].

The Effect of MSCs on Efficacy, Salivary Flow Rate

13 studies included SFR as primary functional outcome [24–29, 31–35, 38, 39]. Eleven studies found a significant increase in SFR after intervention with MSCs compared to other groups (placebo/sham or IR only) [24–29, 32–35, 38], with an overall effect of SMD 6.99 (95% CI: 2.55–11.42), ranging from 1.68 (95% CI: 0.63–2.74) [31] to 25.41(95% CI: 15.22–35.59) [33]. Heterogeneity was $X^2=177.37$ ($p<.001$) and $I^2=93\%$ (95% CI [90–95%]). See Fig. 2.

Subgroup analyses investigating the effect of species, strain, sex, administration route, age in weeks, radiation duration, frequency of treatment, radiation dose and time between radiation to MSC treatment revealed significant differences regarding strain ($p<.001$) and administration route ($p=.01$). The most prominent differences were observed in the strains NOD.SCID-PrckSCID (SMD 22.92, 95% CI: 18.99–26.86) and Wistar (SMD 25.41, 95% CI: 15.22–35.59) compared to C57BL/6 (SMD 2.90, 95% CI: 0.08–5.52), CH3 (SMD 4.99, 95% CI: 1.58–8.39) and Spraque-Dawley (SMD 2.49, 05% CI: 1.81–3.18), while the effect following intraglandular injection was greater than following intravenously injection (SMD 8.59, 95% CI: 2.94–14.24 versus 2.12, 95% CI: 1.20–3.05, respectively). There was no significant effect of species, sex, age in weeks, radiation dose, frequency of treatment or time from radiation to first treatment. Effect of duration of radiation was not possible to assess due to insufficient data. See **Supplementary Results**.

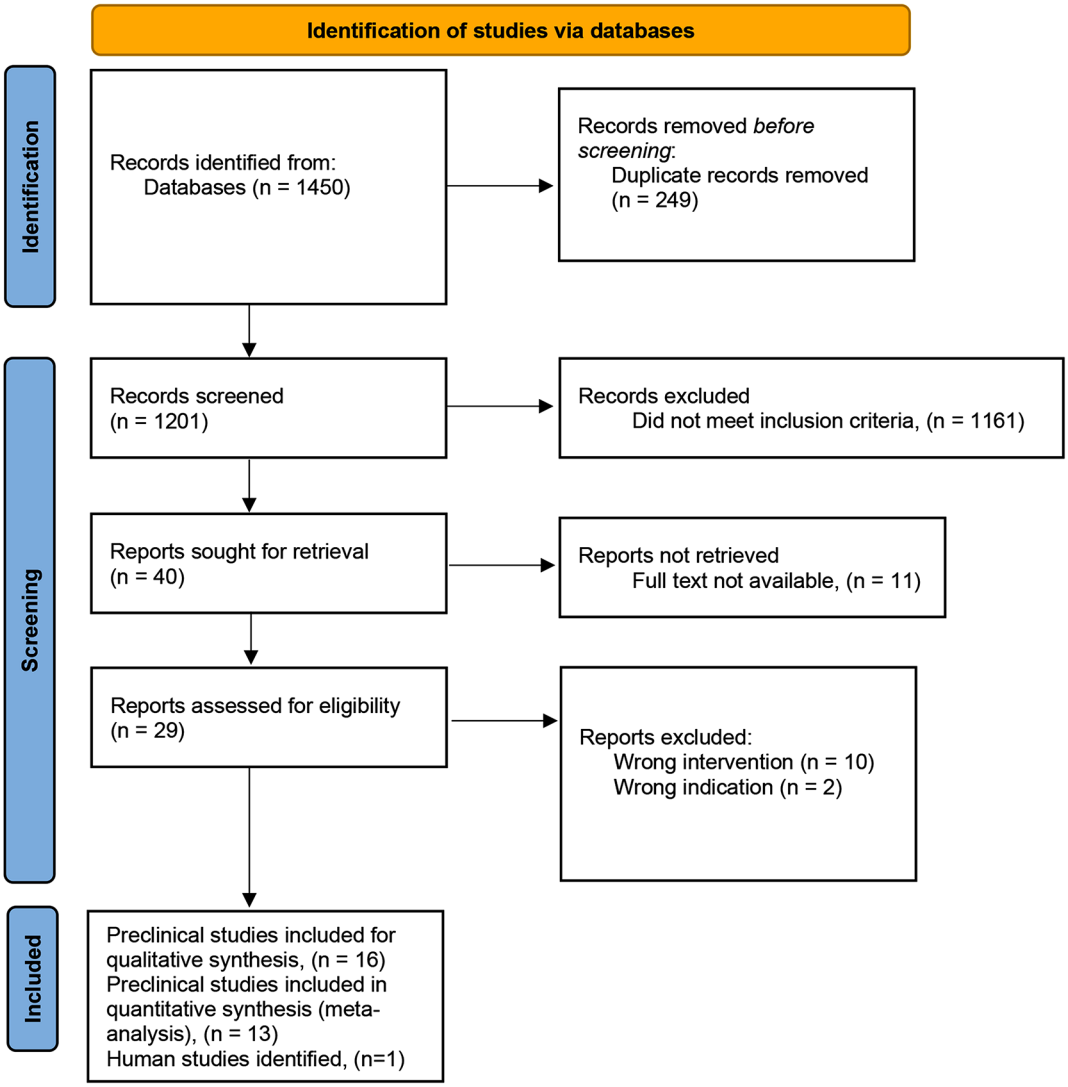

Fig. 1 PRISMA 2020 Statement flow chart of the screening process for the selection of eligible studies

The Effect of MSCs on Salivary Gland Regeneration and Apoptosis

Seven studies reported improvements in acinar tissue [25–27, 29, 31, 32, 35] with more acinar cells [25, 29, 32, 35] and more compact acinar structure [26]. Also, several studies reported less inflammation and fibrosis [25, 28, 30, 33, 35, 36] and increased amylase [27, 28, 31–34, 37–39]. Li et al. and Wang et al. reported intact cellular ultra-microstructure with healthy cell membrane and almost undamaged cytoplasmic organelles [31, 38]. They also found significantly higher proliferative activity, but this was not found by others [27, 28]. Shin et al. found greater expression of SG epithelial cell markers (KRT7 and KRT18) and upregulated structure-related genes (SMR3A, AMY2A5, PRB1, AMY1, CLDN22, PRPMP, AMY1A and AQP5) [35]. Similarly, Choi et al. found higher expressions of AQP5, alfa-SMA and CD31 [33]. Mulyani et al. found increased expression of SDF1-CXCR4 and Bcl-2 genes [37]. Nine studies reported reduction in apoptotic cells [26–33, 38], though Wang et al. did not specify the results [32].

Lim et al. observed anti-alfa-amylase signaling in transplanted MSC(M) suggesting a transdifferentiation into SG epithelial cells [27], but this was not found by Kojima et al. [25].

The Effect of MSCs on Vascular Areas

Several studies reported an increase in vascular areas [25, 27, 29–31]. Furthermore, Kojima et al. localized MSCs in vessel endothelial cells post transplantation five- and ten-weeks post transplantation [25]. Wang et al. also found

Table 1 Study characteristics

Author (Year)	Animal: species, strain and age, gender	Study design	Groups	Irradiation	Days from radiation to MSC treatment	MSC type and concentration	Administration route	Statistical analysis	Functional outcome	Molecular outcome
Lin, C et al. (2011)	Mice, NOD.SCID-*Prck*^{SCID}, four weeks old	Prospective, controlled trial	1. Control group, no IR, no treatment, $n=35$. 2. IR, no treatment, $n=35$. 3. IR, treated with MSC(M), $n=35$. 4. IR, treated with acinar-like cells, $n=35$	Single dose, 15 Gy, from ^{60}Co-source. No QA on radiation delivery was performed.	11 days	10^6 MSC(M) and acinar-like cells, co-cultured from MSC(M).	Intraglandular injection (submandibular gland)	One-way ANOVA and marginal linear regression analysis. Significance level was $P<.05$.	Saliva production in group 3 and 4 significantly increased. Body weight and gland weight significantly higher in groups 3 and 4. Significantly faster rate of recovery in gland weight in group 4, compared to group 3.	No molecular data. However, a significantly higher expression of α-amylase in acinar cells and co-cultured MSC(M) compared to MSC(M) which was not been co-cultured
Kojima, T. et al. (2011)	Mice, C57BL/6, nine weeks old	Prospective, controlled trial	1. Control group, no IR, no treatment, $n=24$. 2. IR, treated with ADSCs in PBS, $n=31$. 3. IR, treated with PBS only, $n=31$.	Single dose, 10 Gy gamma irradiation from ^{137}C source. No QA on radiation delivery was performed.	70 days (10 weeks)	ADSCs 0.1 mL solution of PBS containing 500.000 cells.	Intraglandular injection (submandibular glands)	One-way and two-way ANOVA followed by Fishers PLSD or students *t*-test. Significance level was $P<.01$ or $P<.05$.	Significant increase in SFR in ADSC group compared to sham. 75% function compared to normal group.	Group 2 had more acinar cells and less inflammatory infiltration compared to group 3. Group 2 had significantly more CD31-positivity, and showed a significant increase in levels of HGF, VEGF and several proteins related to angiogenesis.
Jeong, J. et al. (2013)	Rats, Wistar rats, six weeks old, male	Prospective, controlled trial	1. Control group, no IR, no treatment. 2. IR, treated with PBS 3. IR, treated with hSGSCs	Single dose, 25 Gy, dose rate of 2 Gy min^{-1}. No QA on radiation delivery was performed.	1 day	5×10^5 hSGSCs	Intraglandular injection	One-way ANOVA. Significance level of <0.05 was used.	At 60 days, significant increase in SFR and body weight in group 3 compared to group 4.	Group 3 had compact acinar and ductal structure like undamaged rat tissue, whereas group 2 had disrupted acinar structure and numerous vacuoles. Apoptotic cells were observed only in group 2 and not in group 3.
Lim, J. et al. (2013)	Mice, C57BL/6, eight to nine weeks old	Prospective, randomized, controlled trial.	1. Control group, no IR, no treatment, $n=8$. 2. IR, treated with PBS, $n=8$. 3. IR, treated with BM-cMSCs, $n=8$.	Single dose, 15 Gy. No QA on radiation delivery was performed.	1 day	1×10^5 cMSC(M) in 15 µL of PBS.	Intraglandular injection (submandibular glands)	Mann-Whitney test for differences between the two groups. Kruskal-Wallis followed by post hoc Dunns for three groups. Significance level was $P<.05$.	Significant increase in SFR in group 3 compared to group 2. Body weight, gland weight and salivary lag time showed no significant differences.	Significant reduction of apoptosis and increase in microvessel density in group 3 compared to group 2. No significant difference in proliferation activity. Significantly higher mucopolysaccharide and tissue amylase upon histological examination.

Table 1 (continued)

Author (Year)	Animal: species, strain and age, gender	Study design	Groups	Irradiation	Days from radiation to MSC treatment	MSC type and concentration	Administration route	Statistical analysis	Functional outcome	Molecular outcome
Lim, J. et al. (2013)	Mice, CH3, eight to nine weeks old	Prospective, randomized, controlled trial.	1. Control group, no IR, no treatment, $n=20$. 2. IR, no treatment, $n=20$. 3. IR, treated with hAdMSCs, $n=20$.	Single dose, 15 Gy No QA on radiation delivery was performed.	0 days (once weekly for 3 consecutive weeks)	1×10^6 MSC(AT)h.	Intravenously (tail vein)	Mann-Whitney test for differences between two groups. Kruskal-Wallis followed by post hoc Dunns for three groups. Significance level was $P<.05$.	At 12 weeks significant increase in SFR in group 3 compared to group 2. Salivary lag time was significantly longer in group 2 compared to group 1.	A significantly higher fraction of mucin and amylase and reduction in apoptosis in group 3 compared to group 2. No significant differences in proliferative activity.
Xiong, X. et al. (2014)	Rats, Spraque-Dawley, 12 weeks old	Prospective, randomized, controlled trial.	1. Control group, no IR, no treatment, $n=30$. 2. IR, treated with PBS, $n=30$. 3. IR, treated with hADSCs, $n=30$.	Single dose, 18 Gy, dose rate of 300 cGy min^{-1} No QA on radiation delivery was performed.	0 days	1×10^6 MSC(AT)h in 0.1 mL PBS solution.	Intragladular injection (submandibular glands)	One-way ANOVA and Student-Newman-Keuls analysis. Significance level was $P<.05$.	At 24 weeks, significant increase in SFR in group 3 compared to group 2. Group 3 recovered to 71% SFR compared to group 1.	Group 3 had a significantly larger number of acinar cells and PAS-positive acini, significantly higher area of blood vessels and significantly fewer apoptotic cells compared to group 2. Significant increase in mRNA levels of VEGF, HGF, COX-2 and MMP-2 in group 3 compared to group 2.
Chen, Y. and Niu, Z. et al. (2014)	Minipigs, eight months old, female	Prospective, randomized, controlled trial.	1. IR, treated with ADSCs + PRF, $n=5$ 2. IR, treated with ADSCs, $n=5$ 3. IR, treated with PRF, $n=5$ 4. IR, treated with PBS, $n=5$	Single dose, 20 Gy, dose rate of 3 Gy min^{-1}. Only right parotid gland was radiated. No QA on radiation delivery was performed.	28 days (four weeks)	4×10^6 cells ADSCs (autologous),	40 subcutaneous injections points (0.2 mL/point), with 2 cm interval between neighbouring points.	One-way ANOVA and Kruskal-Wallis H test. Significance level was $P<.05$.		Group 1 showed significantly more fat cells, less fibrosis and inflammation compared to group 4. Group 2 and 3 were significantly better compared to group 4. Group 1–3 showed significantly less apoptotic activity compared to group 4 and group 1 showed the least. Group 1–3 showed significantly more neovascular capillary area, compared to group 4 and group 1 showed the most.

Table 1 (continued)

Author (Year)	Animal: species, strain and age, gender	Study design	Groups	Irradiation	Days from radiation to MSC treatment	MSC type and concentration	Administration route	Statistical analysis	Functional outcome	Molecular outcome
An, H. et al. (2015)	Mice, CH3, eight to nine weeks old	Prospective, controlled trial	1. Control group, no IR, no treatment, $n=35$. 2. IR, treated with PBS, $n=35$. 3. IR, treated with hADMSC SEC, $n=35$	Single dose, 15 Gy. No QA on radiation delivery was performed.	0 days (once daily for seven consecutive days)	500 µL MSC(AT)h. Sectretome stemmed from 1×10^5 cell.	Intravenously (tail vein)	Mann-Whitney test, one-way ANOVA followed by Tukey's post hoc test, two-way ANOVA followed by Bonferroni post hoc test and linear regression. Significance level was $P<.05$.	At 16 weeks, significant increase in SFR in group 3 compared to group 2. Salivary lag time was significantly longer in group 3 compared to the other groups. Body and gland weight was significantly higher in group 3 compared to group 2.	Saliva amylase activity was significantly lower in group 2 compared to group 1, while group 3 was not. EGF content in saliva was significantly higher in group 3 compared to group 2. PAS stain for mucin showed significantly larger area of mucin in group 3 compared to group 2.
Li, Z. et al. (2015)	Mice, C57BL/6, eight weeks old	Prospective, randomized, controlled trial	1. IR, treated with ADSCs, $n=10$ 2. IR, treated with PBS, $n=10$ 3. Control, no IR, no treatment, $n=10$	Single dose, 18 Gy No QA on radiation delivery was performed.	0 days (twice a week for six weeks)	1×10^6 cells ADSCs.	Intravenously (tail vein)	ANOVA followed by Tukey's honestly significant difference test. Significance level was $P<.05$.	At eight weeks significant increase in SFR in group 1 compared to group 2. Gland weight was significantly higher in group 1 compared to group 2.	PAS staining of glands showed significant increase in production of mucopolysaccharide, C31 staining showed significantly higher density of microvessels, and significantly higher levels of amylase in group 1 compared to group 2. Significantly higher proliferation activity and reduction of apoptosis activity in group 1 compared to group 2.
Wang, Z. et al. (2016)	Mice, C3H, 8–12 weeks old, female	Prospective, randomized, controlled trial	1. Control group, no IR, no treatment, $n=10$ 2. IR, treated with PBS, $n=10$ 3. IR, treated with ADSCs, $n=10$ 4. IR, treated with PR, $n=10$ 5. IR, treated with ADSCs + PRF, $n=10$	Single dose, 18 Gy No QA on radiation delivery was performed.	84 days (12 weeks)	2×10^5 ADSCs in 100 µL solution of PBS/PRF.	Intragladular injection (submandibular glands). Weekly injections for 3 consecutive weeks.	Mann-Whitney test and one-way ANOVA followed by Tukey's post hoc test. Significance level was $P<.05$.	At 12 weeks. Significant increase in SFR, body weight and gland weight in group 3, 4 and 5 compared to group 2.	Group 5 showed significantly more acinar cells, more amylase area compared to groups 2,3 and 4. Group 5 showed significantly more microvessels, compared to group 2 and 4, but not group 3. Group 5 showed less apoptotic activity and more proliferation activity compared to group 2.

Table 1 (continued)

Author (Year)	Animal: species, strain and age, gender	Study design	Groups	Irradiation	Days from radiation to MSC treatment	MSC type and concentration	Administration route	Statistical analysis	Functional outcome	Molecular outcome
Wang, Z. et al. (2017)	Miniature pigs, eight months old, female	Prospective, randomized, controlled trial	1. Control group, no IR, no treatment, $n=5$ 2. IR, treated with ADSCs, $n=5$ 3. IR, treated with PBS, $n=5$	Single dose, 20 Gy, dose rate was 3.2 gy min^{-1}. Only right parotid gland was radiated. No QA on radiation delivery was performed.	0 days (un-clear?)	4×10^6 ADSCs	Intraglandular injection at 40 different injection points, 0.2 mL per injection. Repeated twice per week for 6 weeks.	ANOVA followed by Tukey's honestly significant difference test. Significance level was $P<.05$.	At 3 months significant increase in SFR and gland weight in group 2 compared to group 3. There was no significant difference in gland weight between group 1 and 2.	Amylase levels was significantly higher in group 2 compared to group 3. The number of PAS-positive acinar cells was significantly higher in group 2 compared to group 3. Group 2 showed less fibrosis and more vascularization than group 3.
Choi, J. et al. (2018)	Mice, C3H, eight to nine weeks old.	Prospective, randomized, controlled trial	1. Control group, no IR, no treatment, $n=28$ 2. IR, treated with PBS, $n=28$ 3. IR, treated with porcine SIS, $n=28$ 4. IR, treated with AdMSC, $n=28$ 5. IR, treated with AdMSC + SIS, $n=28$	Single dose, 15 Gy No QA on radiation delivery was performed.	0 days	1×10^5 cells MSC(AT) in 20 µL solution of respective carrier.	Intraglandular injection (submandibular glands)	T-test, one-way ANOVA followed by Tukey's post hoc test. Significance level was $P<.05$.	At 16 weeks, significant increase in SFR in group 5 compared to group 2. Group 4 and 5 showed significantly lower lag-time compared to group 2.	Group 4 and 5 showed significantly higher levels of EGF and amylase compared group 2. Group 4 and 5 showed significantly less fibrosis than group 2 and 3. Only group 5 showed increased mucin production, compared to group 2 and 3. Group 4 and 5 showed significant anti-apoptotic effects and increased ROS scavenging effects compared to group 2.
Shin, H. et al. (2018)	Mice, C3H, six weeks old, female	Prospective, randomized, controlled trial	1. Control group, no IR, no treatment, $n=6$ 2. IR, treated with PBS, $n=6$ 3. IR, treated with $SGSC^{2D}$, $n=6$ 4. IR, treated with $SGSC^{3D}$, $n=6$	Single dose, 15 Gy No QA on radiation delivery was performed.	28 days (four weeks)	2×10^5 $SGSC^{3D}$ $SGSC^{2D}$ in 10 µL solution of PBS.	Intraglandular injection (submandibular glands)	Mann-Whitney test, one-way ANOVA followed by Tukey's post hoc test, two-way ANOVA followed by Bonferroni post hoc test, as well as linear regression. Significance level was $P<.05$.	At 16 weeks, significant increase in SFR, body and gland weight and lower lag time in group 3 and 4 compared to group 2. Significant increase in SFR, body and gland weight and lower lag time in group 4 compared to group 3	Group 3 and 4 showed significantly higher amylase activity and higher levels of EGF compared to group 2. Group 4 showed significantly higher amylase activity and levels of EGF compared to group 3. Group 3 and 4 showed significantly higher expression of genes related to salivary gland function and growth factors compared to group 2. Group 4 showed significantly higher expression compared to group 3, however by a smaller relative margin.

Table 1 (continued)

Author (Year)	Animal: species, strain and age, gender	Study design	Groups	Irradiation	Days from radiation to MSC treatment	MSC type and concentration	Administration route	Statistical analysis	Functional outcome	Molecular outcome
Shin, H. et al. (2018)	Mice, C3H, six weeks old.	Prospective, randomized, controlled trial	1. Control group, no IR, no treatment, $n=4$ 2. IR, treated with PBS, $n=4$ 3. IR, treated with hADSC^{NMX}, $n=4$ 4. IR, treated with hADSC^{HPX}, $n=4$	Single dose, 15 Gy. No QA on radiation delivery was performed.	28 days (four weeks)	2×10^5 MSC(AT)h^{HPX} and MSC(AT)h^{NMX} in 10 µL solution of PBS.	Intragladular injection (submandibular glands)	Mann-Whitney test, one-way ANOVA followed by Tukey's post hoc test, two-way ANOVA followed by Bonferroni post hoc test. Significance level was $P<.05$.	At 12 weeks significant increase in SFR, **amylase activity**, body and gland weight in group 3 and 4 compared to group 2, and significantly higher in group 4 compared to group 3. Lag time was significantly shorter in group 3 & 4 compared to group 2.	Significantly more fluorescently labelled MSC(AT)h^{HPX} cells compared to MSC(AT)h ^{NMX} cells in random fields. The density of PAS-positive acinar cells was significantly higher in group 3 and 4 compared to group 2, and significantly higher in group 4 compared to group 3. Significantly less fibrosis in group 3 and 4 compared to group 2. EGF levels were higher in group 3 and 4 compared to group 2.

Table 1 (continued)

Author (Year)	Animal: species, strain and age, gender	Study design	Groups	Irradiation	Days from radiation to MSC treatment	MSC type and concentration	Administration route	Statistical analysis	Functional outcome	Molecular outcome
Elsaadany, B. et al. (2019)	Rats, albino, three to four months old, male	Prospective, randomized, controlled trial	1. Control group, no IR, no treatment, $n=6$ 2. IR, treated with PBS, $n=6$ 3. IR, treated with MSC(M), $n=6$	Single dose, 13 Gy. No QA on radiation delivery was performed.	0 days	1×10^5 MSC(M) in 0.2 mL PBS solution.	Intraglandular injection.	ANOVA followed by Tukey's post hoc test. Significance level was $P<.05$.	No functional outcome parameter.	The acini diameter was significantly larger in group 3 compared to group 2. Group 3 showed significantly less fibrosis compared to group 2. Group 2 showed necrosis of the ductal lining epithelium, which was less commonly observed in group 3, however no significantly different data was reported.
Mulyani, S. et al. (2019)	Rats, Wistar, three to four months old, male	Prospective, randomized, controlled trial	1. Control group, no IR, no treatment, $n=10$ 2. IR, no treatment, $n=10$ 3. IR, treated with $MSC(M)^{NMX}$, $n=10$ 4. IR, treated with $MSC(M)^{HPX}$, $n=10$	Single dose, 15 Gy. No QA on radiation delivery was performed.	1 day	$MSC(M)^{HPX}$ and $MSC(M)^{NMX}$. Dose not specified.	Not specified	Normality test and MANOVA test. Significance level was $P<.05$.	At 7 days, group 4 showed a significantly higher expression of amylase compared to group 3. Group 3 and 4 showed significantly higher levels compared to group 2.	The expression of SDF1-CXCR4 and Bcl-2 was significantly higher in group 4 compared to group 3.

Abbreviations: ADSC, adipose-derived stromal cells; ANOVA, analysis of variance; Bcl-2, B-cell lymphoma 2; COX-2, cyclooxygenase-2; EGF, epidermal growth factor; HGF, hepatocyte growth factor; hADSC, human adipose-derived stromal cells; hSGCs, human salivary gland stem cells; IR, irradiation; MANOVA, multivariate analysis of variance; MMP-2, matrix-metalloproteinase-2; MSC(AT)h, human adipose-derived mesenchymal stem cells; MSC(AT)h SEC, human adipose-derived mesenchymal cell secretome; $MSC(AT)h^{HPX}$, human adipose-derived mesenchymal stem cells incubated in a hypoxic environment; $MSC(AT)h^{NMX}$, human adipose-derived mesenchymal stem cells incubated in a normoxic environment; MSC(M), bone marrow-derived clonal mesenchymal stem cells; $MSC(M)^{HPX}$, bone marrow-derived mesenchymal stem cells incubated in a hypoxic environment; $MSC(M)^{NMX}$, bone marrow-derived mesenchymal stem cells incubated in a normoxic environment; PBS, phosphate-buffered saline (PBS) solution; PAS, Periodic acid Schiff; PRF, platelet-rich fibrin; SGSC2D, human parotid gland 2D monolayer cultured human salivary gland stem cells; SGSC3D, 3D spheroid microwell cultured human salivary gland stem cells; SDF1-CXCR4, stromal cell-derived factor 1-C-X-C chemokine receptor type 4; SFR, salivary flow rate; SIS, small intestine submucosa; VEGF, vascular endothelial growth factor; QA, quality assurance

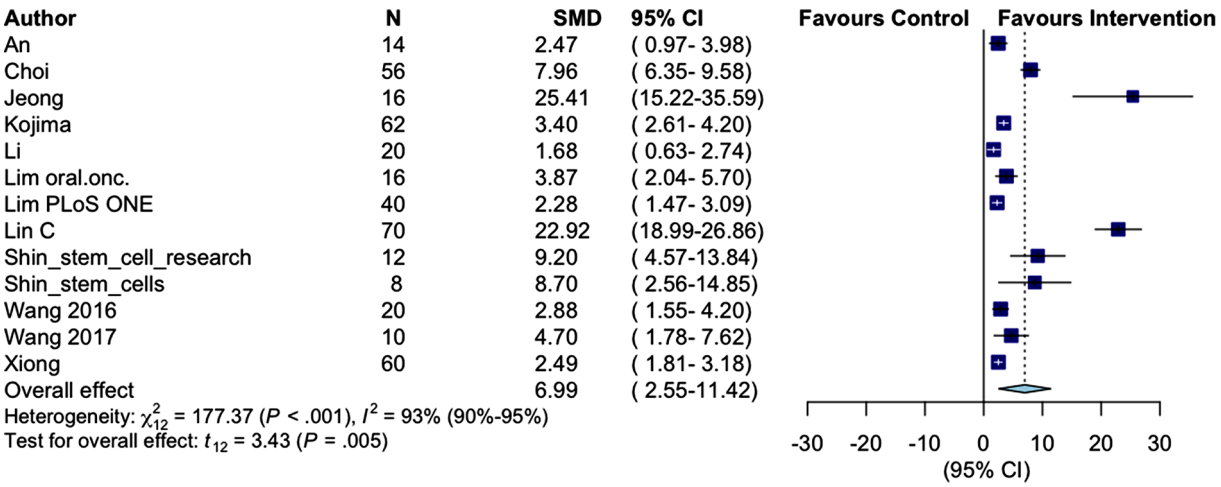

Fig. 2 Random-effects meta-analysis of the overall effect on SFR following MSC therapy. SFR: salivary flow rate; SMD: standardized mean difference; MSC: mesenchymal stem cell

improvements in vascular areas, but only for the intervention group receiving both MSCs and platelet-rich-fibrin (PRF) [38].

The Paracrine Effects of MSCs

Five studies reported on the paracrine effect of MSC treatment [25, 29, 33–35]: Xiong et el. found increased mRNA levels VEGF, HGF and COX-2 [29]; Shin et al. found higher expression of the paracrine factors BDNF, GDNF, EGF, IGF1 and NGF [34]; Shin et al. found greater expression of the growth factor FGF10 [35]; Kojima et al., found increased expressions of HGF, VEGF, COX-2 and MMP-2 [25] and Choi et al., found increased levels of EGF [33].

Homing of Systemically Transplanted MSCs

Both Li et al. and Lim et al. found that systematically transplanted MSCs could be identified in the salivary glands post transplantation [28, 31]. An et al. also administered the therapy intravenously but did not report on homing to the SG post transplantation [39].

Platelet-Rich Fibrin in Addition to MSCs

Two studies also investigated MSC + PRF [30, 38]. Chen et al. found the MSC + PRF group had significantly improvement on soft tissue defects [30], while Wang et al. found that MSC + PRF had increased levels of acinar cells, amylase, microvessels and proliferative activity and reduced levels of apoptotic cells [38].

The Effect of MSCs on Efficacy and Safety in Human Studies

One human study investigated the intervention of intraglandular autologous MSCs(AT) in a randomized, placebo-controlled phase I/II study ($n = 30$) [19]. The study found MSC treatment to be safe and reported a significant increased UWS (50% after four months, $p = .003$) including improvements in patient-reported outcomes in the group receiving MSCs. Also, a significant increase in serous gland tissue, improvements in saliva composition, and a decrease in connective were observed. See **Supplementary Results**.

Risk of Bias and Quality Assessment

The preclinical in vivo studies were assessed using SYRCLEs risk of bias assessment tool with nine questions to determine potential biases [22]. All studies involved a risk of bias, especially regarding selection bias as none of the studies reported how the randomization of intervention/control groups was performed [24–39]. Also, all studies revealed a high risk of performance bias, since none of the included studies used random housing or blinding of interventions/investigators [24–39]. All studies, except Mulyani et al., had a low risk of bias concerning attrition bias and reporting bias. Seven studies had a low risk of detection bias since they reported blinding of the outcome assessment [27, 28, 32, 33]: (1) histological examination [26, 35] (2) immunohistochemical evaluation [27, 31, 32, 37] (3) evaluation of apoptotic cells [26, 27, 32, 37, 38] (4) evaluation of cytoprotective effects (AQP5, CD31, alfa-SMA, c-Kit) [38]. Nine studies did not report blinding [24–26, 29–31, 34, 35, 37]. See Fig. 3.

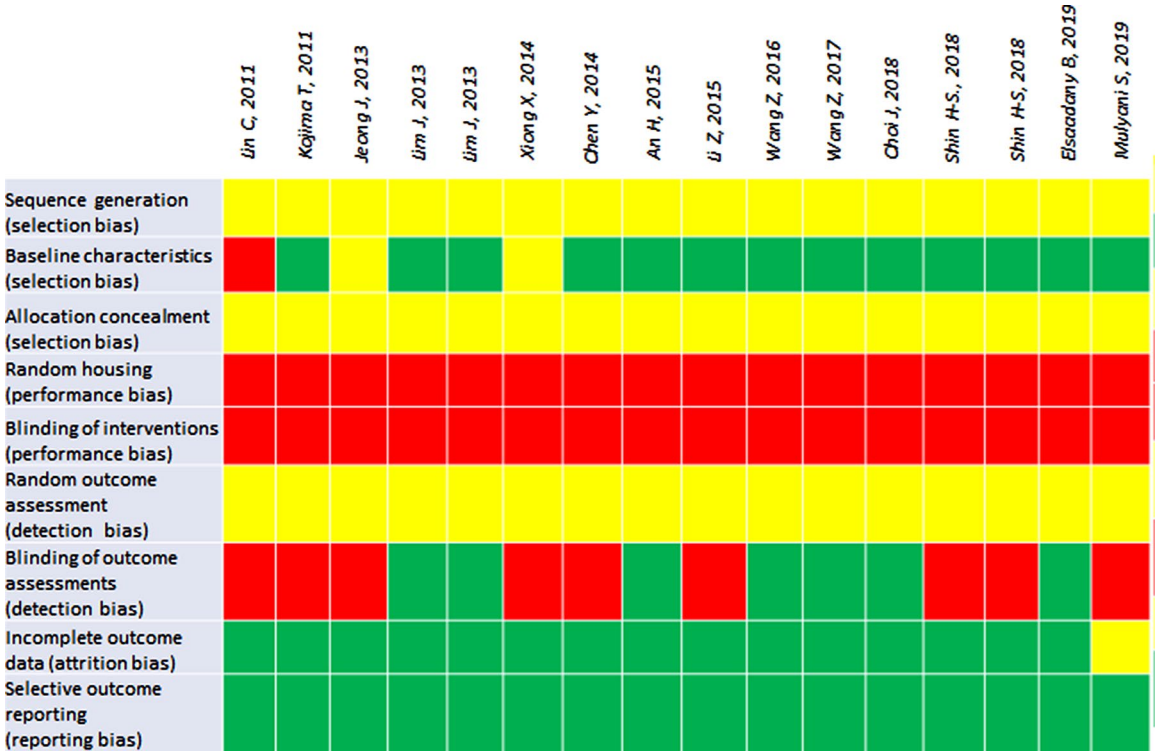

Fig. 3 SYRCLEs Risk of bias assessment tool. *Green* = low risk of bias, *Yellow* = unclear risk of bias, *Red* = high risk of bias

The quality of the included studies was assessed using the ARRIVE guidelines [21]. Highest score given was 9 [27, 28, 32, 33, 36, 38] and lowest score given was 5 [26]. Most studies revealed high quality with ≥8 points [25, 27–29, 31–36, 38, 39], but 4 studies had a total score of ≤7 [24, 26, 30, 37]. All studies except Jeong et al. reported sufficiently regarding study design and sample size [24, 25, 27–38]. All studies reported sufficiently on outcome measures and results and all studies were randomized, though none reported on the randomization method used and none reported how they included/excluded animals [24–39]. One study failed to report sufficiently on statistical method [24], one on experimental animals [30] and two studies failed to report sufficiently on experimental procedures [26, 37]. See **Supplementary Results.**

Discussion

In this systematic review and meta-analysis, MSC therapy demonstrated a significant effect on SG function following radiation-induced gland damage in preclinical in vivo studies. The treatment proved to be safe, with no reported adverse reactions.

We found that MSC therapy had a significant impact on the SG functioning with a significant increase in the SFR. However, there was a high heterogeneity among included studies with differences in MSC origin, species, strains, age, radiation dose, administration route of MSC therapy, frequency of treatment and time between radiation and first treatment. The impact on SFR was significantly associated with strain and administration route. The most pronounced effect on SFR was observed when MSCs were administered intraglandular compared to systemic transplantation. Whether the effect of MSC therapy varies by strain is difficult to conclude, as only one study included Wistar [26], and one study NOD.SCID-PrckSCID [24], which both exhibited the most significant effect.

All studies but one, by Lin et al., reported elements of SG remodeling properties such as: increased density of acinar cells, more compact acinar structure, increased levels of amylase, decreased inflammation and fibrosis. If MSC therapy induces a higher proliferative activity remains uncertain. While Shin et al. reported such an effect, it was not confirmed by other studies [26, 27]. As possible modes of action, upregulation in epithelial markers (KRT7 and KRT18) and structure-related genes (SMR3A, AMY2A5, PRB1, AMY1, CLDN22, PRPMP, AMY1A, AQP5, AQP5, alfa-SMA and CD31) was identified [33, 35], while another study by Mulyani et al. found upregulation in genes encoding for proteins involved in cell migration, survival and differentiation (SDF1-CXCR4 and Bcl-2) [37].

This study also indicates that increased blood vessel regeneration and paracrine functioning participate in the

tissue repair and restoration of gland damage. Several studies reported an increase in vascular areas [24, 26, 28–30, 32]. A wide range of growth factors (VEGF, HGF, COX-2, BDNF, GDNF, EGF, IGF1, NGF, FGF10 and MMP-2) contributing to various aspects of regeneration including angiogenesis, neural regeneration and cellular proliferation were identified – ultimately supporting the repair of damaged glandular tissues.

The severity of radio-induced salivary gland damage is influenced by several factors, with radiation mean dose being a critical one [40]. The delivery of radiotherapy was greatly standardized and administered as a single dose in all the included studies, which does not resemble the clinical radiotherapy regimens for head and neck cancer which are patient-specific and often fractionated across several weeks. This is important to keep in mind, when translating the findings from this systematic review to a clinical setting. Prolonged exposure to radiation leads to cumulative damage, but unfortunately, we could not further investigate the relation between radiation duration and effect of MSC therapy due to insufficient data. The time from radiotherapy to administration of MSC might also be important, since especially the decreased levels of apoptotic cells found in several studies [26–28, 38], indicates that MSC therapy could be protective in the acute phase of radiotherapy. The timing of MSC administration varied across the studies from 0 days [28, 29, 31–33, 36, 39] to 12 weeks [25] post radiotherapy, but we observed no impact of the time from radiation to the initial MSC treatment.

Two studies that investigated the effect of intravenously administered MSC therapy also reported on homing to the SGs post transplantation, both identifying MSCs in the SGs [28, 31]. However, large-scale studies are required to further investigate the migration and homing following systemic transplantation.

PRF is a platelet-rich regenerative therapy containing a variety of growth factors and is known to promote cell proliferation [41]. Two studies investigated if additional PRF treatment improved SG function [30, 38]. Chen et al. found the MSC + PRF group had significantly improvement on soft tissue defects, but the groups were small, $n = 5$ [30]. Wang et al. found that interventions with MSC, PRF or MSC + PRF improved SFR, gland and body weight, but MSC + PRF performed better regarding the regenerative outcomes [38].

In addition, the preclinical in vivo studies included in the meta-analysis, we identified one human, phase I/II, randomized, placebo-controlled clinical trial. The study found no serious adverse events and a significant effect on unstimulated SFR four months post MSC(AT) therapy in the treated group [19]. This is also supported by a recent study by Lynggaard et al. [16]. However, the long-term effects of intraglandular MSC therapy in humans remain divergent [18, 42].

As a possible mode of action, Lynggaard et al. also investigated the regenerative effects of intraglandular allogeneic MSC(AT) therapy on the salivary proteome [43]. They observed an increase in proteins associated with tissue regeneration post transplantation, yet the salivary proteome did not return to a healthy state when compared to healthy controls [43].

This review is limited by the heterogeneity of methodologies and limited long-term data, hindering definite conclusions. The included studies varied in relation to included species, strains, origin of MSCs, delivering methods, radiation and follow-up regimen and study design. Prospects lie in optimizing challenges related to standardization of MSC therapy such as delivery methods, origin, and refining dosage protocols. Also, the radiotherapy regimens in the preclinical in vivo models were standardized and did not mirror those used in head and neck cancer patients. This lack of resemblance could potentially influence the effectiveness of MSC therapy in a clinical setting.

In conclusion, this systematic review and meta-analysis showed a significant effect of MSC therapy for restoring SG functioning and regenerating SG tissue following radiotherapy in preclinical in vivo studies. No serious adverse events were identified and intraglandular transplantation performed better effect than systemic transplantation. MSC therapy holds significant therapeutic potential in the treatment of radio-induced xerostomia and hypofunction, but comprehensive, randomized, clinical trials in humans are required to ascertain their efficacy in a clinical setting.

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Authors Contribution **ALFC**: investigation, data curation, writing original draft preparation, visualization **AG**: data curation, review and editing **CDL**: Conceptualization, methodology, review and editing **PMJ**: review and editing **BF**: investigation, review and editing **CH**: investigation, review and editing **BSK**: review and editing **RTL**: formal analysis, review and editing **CG**: conceptualization, methodology, review and editing **KKJ**: conceptualization, review and editing **AFN**: review and editing **CVB**: Conceptualization, methodology, review and editing.

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Code Availability Not applicable.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent for publication Not applicable.

Conflict of interest C.D. Lynggaard, C. Grønhøj, and C. von Buchwald are co-inventors on a patent application PCT/EP2020/053878; the patent is owned by Rigshospitalet, Copenhagen University Hospital and the University of Copenhagen.

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Supplementary Results

Table 1. Random-effects model sub analyses on categorical heterogeneity factors.

Subgroup analysis	Classifications	No. of studies	SMD	95% CI	p-value*
Species	Mice	10	6.3	1.8-10.8	0.63
	Rats	2	13.4	-132.0-158.7	
	Miniature pigs	1	4.7	1.7-7.6	
Strain	NOD.SCID-PrckSCID	1	22.9	19.0-26.9	<0.001*
	C57BL/6	3	2.9	0.1-5.7	
	Whistar	1	25.4	15.2-35.6	
	CH3	6	5.0	1.6-8.4	
	Spraque-Dawlay	1	2.5	1.8-3.2	
	Missing	1	4.7	1.8-7.6	
Sex	Female	9	4.3	2.1-6.4	0.47
	Male	2	13.4	-132.0-158.7	
	Unknown	2	13.3	-107.7-134.3	
Administration route	Intraglandular	10	8.6	2.9-14.2	0.01*
	Intravenously	3	2.1	1.2-3.0	

*significant value.

Table 2. Meta-regression sub analyses on continuous heterogeneity factors

Subgroup	Estimate	95% CI	p-value*
Age, weeks	-0.3	-0.9-0.3	0.3
Radiation dose, Gy	-0.7	-0.7-2.1	0.3
Frequency of treatment	-0.6	-1.7-0.5	0.2
Time between radiation and first treatment	0.0	-0.2-0.1	0.7

*significant value.

Table 3. Human study characteristic.

Author (year)	Study design	Groups	Irradiation	Days from radiation to MSC treatment	MSC type, concentration and administration route	Statistical analysis	Functional outcome	Molecular outcome
Grønhøj, C. et al. (2018)	Prospective, randomized, blinded, controlled phase 1/2 trial	1. placebo, n = 30 2. MSCs(AT), n = 30	Mean Gy dose to single gland between 11.4 Gy and 71 Gy. 27 patients received both RT and chemotherapy, 3 received only RT.	Between 2.8 and 6.5 years. Median interval was 4.1 years.	MSC(AT)h (autologous). Dose was 2.8 x 10^6 cells x the volume of the gland (cm^3). Intraglandular injection, ultrasound guided (submandibular glands)	Sample size was calculated using a non-paired *t* test. Within-group comparisons were performed with the Wilcoxon signed-rank test, and between-group comparisons were performed with the Mann-Whitney U test. Nonparametric statistics was evaluated by Shapiro-Wilks tests. Significance level was P < .05.	Unstimulated whole SFR was significantly increased in group 2 compared to baseline at both one-month and four months after treatment. Control group did not see this increase. The net scores between the two groups were similar. Group 2 showed significant improvements in patient reported outcome measures such as VAS score and xerostomia questionnaire regarding thirst and oral dryness. No adverse events in group 2 at 1 year.	19 samples were evaluated, 11 were deemed not suitable for evaluation. A significant increase in serous gland tissue in group 2 was found compared to group 1. No significant differences was observed in the fractions of mucinous to serous tissue, mucinous tissue, glandular tissue adipose tissue.

Figure 2. Evaluation of reporting quality according to ARRIVE guidelines. *: 1=Study design; 2= Sample size; 3= Inclusion and exclusion criteria; 4= Randomization, **none of the studies reported randomization method; 5=Blinding; 6=Outcome measures; 7=Statistical methods; 8=Experimental animals; 9=Experimental procedures; 10=Results. One point was given if the criteria was sufficiently fulfilled, maximum total quality score possible was 10.

Author (Year)	1*	2*	3*	4*	5**	6*	7*	8*	9*	10*	Total quality score
Lin, C et al. (2011)	1	1	0	1**	0	1	0	1	1	1	7
Kojima, T. et al. (2011)	1	1	0	1**	0	1	1	1	1	1	8
Jeong, J. et al. (2013)	0	0	0	1**	0	1	1	1	0	1	5
Lim, J. et al. (2013, oral)	1	1	0	1**	1	1	1	1	1	1	9
Lim, J. et al. (2013, PLoS ONE)	1	1	0	1**	1	1	1	1	1	1	9
Xiong, X. et al. (2014)	1	1	0	1**	0	1	1	1	1	1	8
Chen, Y. and Niu, Z. et al. (2014)	1	1	0	1**	0	1	1	0	1	1	7
An, H. et al. (2015)	1	1	0	1**	1	1	1	1	1	1	8
Li, Z. et al. (2015)	1	1	0	1**	0	1	1	1	1	1	8
Wang, Z. et al. (2016)	1	1	0	1**	1	1	1	1	1	1	9
Wang, Z. et al. (2017)	1	1	0	1**	1	1	1	1	1	1	9
Choi, J. et al. (2018)	1	1	0	1**	1	1	1	1	1	1	9
Shin, H. et al. (2018)	1	1	0	1**	0	1	1	1	1	1	8
Shin, H. et al. (2018)	1	1	0	1**	0	1	1	1	1	1	8
Elsaadany, B. et al. (2019)	1	1	0	1**	1	1	1	1	1	1	9
Mulyani, S. et al. (2019)	1	1	0	1**	0	1	1	1	0	1	7

Title

Long-term effectiveness and safety of mesenchymal stromal cell therapy for radiation-induced hyposalivation in head and neck cancer survivors: A randomised, phase-2, trial

Authors

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Conflict of Interest

Charlotte Lynggaard, Christian Grønhøj, and Christian von Buchwald are co-inventors on a patent application for “Stem cell therapy for patients with salivary gland dysfunction”, PCT/EP2020/053878. The patent is owned by Rigshospitalet, Copenhagen University Hospital and University of Copenhagen.
Jens Kastrup, Annette Ekblond, and Mandana Haack-Sørensen hold a patent application for the investigated stem cell product “Stem cell therapy based on adipose-derived stem cells”, WO2017068140. None of the other co-authors declare conflicts of interest.

Funding

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Keywords

Mesenchymal Stem/stromal Cells; Mesenchymal Stromal Cells; MSC; Xerostomia; hyposalivation, Radiotherapy; Head and Neck cancer

Translational Relevance

The most common side effect of radiotherapy for head and neck cancer is radiation-induced hyposalivation, which impacts swallowing, the oral health and the quality of life in cancer survivors. Currently, only options for symptomatic relief exists, but mesenchymal stromal cells (MSCs) have been suggested as a regenerative treatment. We have previously shown that intraglandular treatment with MSCs hold significant potential in restoring the salivary gland function, and this study is the first long-term results of a randomised, placebo-controlled phase II trial investigating allogeneic MSCs for radiation-induced hyposalivation. The study revealed that MSC treatment improved the subjective feeling of dry mouth. However, treatment with ASCs was not superior to placebo in restoring salivary gland function evaluated by objective salivary flow rate measurements. This study underscores the clinical potential of MSC treatment in cancer rehabilitation, however, identification of patient subgroups along with implementation of repeated MSC treatment may contribute to refining therapeutic strategies.

Abstract

Background: The long-term effect of adipose-derived mesenchymal stromal cells (ASCs) to restore radiation-induced salivary gland hypofunction in previous head and neck cancer patients have not been validated in larger, randomised settings.

Methods: The study was the 12-months follow-up of a randomised, phase-2 trial, including patients with hyposalivation. Patients were randomised to receive allogeneic ASCs or placebo in the submandibular glands. Primary endpoint was unstimulated whole saliva (UWS) followed by stimulated whole saliva, patient-reported outcomes (European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, Head and Neck Module and the Xerostomia Questionnaire) and safety (serious adverse events and immune response).

Results: Of the 120 enrolled patients, 117 (97.5%) were assessed at 12 months. Treatment with ASCs did not increase UWS compared to placebo: the increase in UWS was 0.02 mL/min (95% CI 0.01 to 0.04) in the ASC group and 0.02 mL/min (95% CI 0 to 0.03) in the placebo group, p=0.56. ASCs significantly reduced the symptom burden for dry mouth with -10.07 units (95% CI -13.39 to -6.75) compared to -4.15 units (95% CI -7.46 to -0.84) in the placebo group, p=0.01. Compared to placebo, ASCs did not improve sticky saliva (-9.27 vs. -4.55 units, p=0.13), swallowing (-4.50 vs. 3.49 units, p=0.5) or xerostomia -3.12 vs. -2.74 units, p=0.82). Treatment was safe and associated with a transient immune response.

Conclusion: Intraglandular ACS therapy in the submandibular glands significantly relieved subjective dry mouth symptoms. Both ASCs and placebo increased UWS, but ASCs did not prove superior to placebo in restoring salivary gland function, based on salivary flow rate.

Introduction

Head and neck cancer (HNC) affects globally more than 900,000 individuals each year, and with an increasing incidence[1]. Most patients are treated with radiation therapy (RT) either as a single modality or in combination with other treatment modalities[2,3]. Despite a reduction in salivary gland radiation dose following introduction of intensity-modulated RT (IMRT), the salivary glands are still often affected[4,5]. The salivary glands are especially susceptible to radiation which induces both acute and chronic salivary gland damage, including inflammation, fibrosis, and loss of acinar and progenitor cells, ultimately leading to salivary gland hypofunction[6,7]. The most common side effect of RT is hyposalivation and xerostomia, or dry mouth syndrome, and affects more than 80% of head and neck cancer patients treated with RT[8]. Xerostomia impacts not only the quality of life but also the health of head and neck cancer survivors: hyposalivation is associated with an increased risk of oral infections, discomfort, pain, swallowing difficulties, impaired speech, and sleep disturbances [9–12]. Current treatment modalities for xerostomia only provide symptomatic relief[13], emphasizing the urgency for novel therapeutic opportunities.

Mesenchymal stromal cells (MSCs) have been investigated as a possible therapeutic and disease-modifying treatment to restore radiation-induced salivary gland damage and hypofunction[14]. This is attributed to their regenerative potential which includes mesodermal differentiation, efferocytosis, and microenvironmental reorganization as well as trophic properties encompassing immunosuppression, antifibrosis, and angiogenetic effects[15,16]. The mode of action is not fully understood but they are assumed to function through a “hit and run” mechanism[17] while also escaping the innate immune system[18] enabling an easily accessible use of “off the shelf” donor MSCs. Intraglandular MSC therapy has shown to improve quality of life and enhance the salivary flow rate (SFR) in both preclinical, animal studies[19] and in humans[20–23]. We recently conducted a randomised, clinical trial, demonstrating that intraglandular allogeneic ASC therapy was safe and was associated with an increase of 38% in saliva production, although not superior to placebo 4 months following intervention[22]. Also, long-term outcomes indicate a persistent effect, however, this has not been validated in a larger, randomised trial setting[24,25].

Therefore, the aim of this phase 2 trial was to evaluate the long-term efficacy and safety of intraglandular treatment with allogeneic ASCs for radiation-induced hyposalivation in previous patients with HNC from our randomised clinical trial [22].

Materials and methods

Trial design

The MESRIX-III trial was a single-centre, investigator-initiated, randomised, placebo-controlled, double-blinded trial conducted at the Department of Otorhinolaryngology, Head and Neck Surgery & Audiology, University Hospital of Copenhagen. Patients were followed for one year and the present study constitutes the long-term follow-up of the trial[22]. The trial was approved by the National Ethics Committee (1802872), The Danish Data Protection Agency (P-2020-1164), and the Danish Medical Agency (EudraCT: 2018-000348-24). The study protocol was previously published[26] and registered at ClinicalTRails.gov (NCT04776538). The Good Clinical Practice unit (GCP) at University of Copenhagen monitored the trial. In accordance with the Helsinki Declaration included patients provided both verbal and written consent to participate.

Participants

Patients eligible for inclusion encompassed: 1) age between 18-75 years 2) previously RT treated for head and neck cancer 3) clinically hyposalivation with unstimulated whole saliva flow rate (UWS) 0.05-0.25 mL/min 3), and a minimum of two years since diagnosis or recurrence [22,26]. Patients were excluded if they received xerogenic medicine, had other salivary gland disease, other cancer within the last four years, recent alcohol abuse or smoking history, penicillin or streptomycin allergy, pregnancy, or breastfeeding.

Interventions

Patients were randomised 1:1 to receive intraglandular injection with ASC therapy or injection with a placebo solution in both submandibular glands. Into each gland, an amount of 0.5 mL was injected ultrasound guided without using local anaesthesia, corresponding to a dose of 25 x 10^6 cells for patients receiving ASCs per gland. The injections were, as far as possible, distributed in a fan-shaped pattern within each gland[22]. The Cardiology Stem Cell Centre (CSCC) provided the ASCs according to good manufacturing practice (GMP) from three healthy donors [27,28], with each vial containing cells from one individual donor. Placebo consisted of the freezing media for ASCs CryoStor10 (BiolifeSolutions) with 10% dimethyl sulfoxide (DMSO). The interventions of the study are previously well described[22,26].
UWS and stimulated whole saliva flow rate (SWS) was evaluated by sialometry. Patient-reported outcomes on quality of life were evaluated by two questionnaires, both the xerostomia questionnaire (XQ) and the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, Head and Neck Module (EORCT QLQ-H&N35) domains for dry mouth (HNDR), sticky saliva (HNSS), and swallowing (HNSW). Immune response was evaluated by the development of de novo human leukocyte antigen (HLA) donor specific antibodies. Safety was evaluated by serious adverse events (SAEs). The assessments of the study are previously well described[22,26].

Objectives and Outcomes

The primary objective of this study was to assess the effectiveness of ASCs compared to placebo on UWS, measured as change in mL/min from baseline to 12 months follow-up.

Secondary objectives were to assess ASCs compared to placebo from baseline to 12 months on the following: 1) the effectiveness measured as change in SWS in mL/min 2) the impact on patient-reported outcomes (XQ, EORCT QLQ-H&N35 domains: HNDR, HNSS, HNSW) measured as change in sum score 3) the safety measured as development of SAEs and 4) immune response measured as development of donor specific antibodies. An increase or decrease in donor specific antibodies was defined as a change in mean florescence intensity sum of donor specific antibodies $\geq$ 3000.

Sample size, randomisation, and blinding

A total of 120 patients were included yielding a power of 80% and an alpha of 0.05. Patients were randomised 1:1 according to their treatment order and a predetermined block randomisation code ensured no treatment clustering. The sample size and randomisation are previously well described [22,26]. The trial was kept blinded to sponsor, investigators, and research staff until the 4 months follow-up as previously described[22,26]. The 12 months follow-up was conducted unblinded to treatment allocation to both study personnel and participants.

Statistical methods

Prior to conducting the analysis, a statistical analysis plan was completed, **Supplementary Statistical Analysis Plan**. The primary analyses were based on the intention to treat (IIT) principle; participants allocated to a treatment group (ASC and Placebo, respectively) was followed up, assessed, and analysed as members of that group, irrespective of their adherence to the planned course of treatment (i.e., independent of withdrawals and cross-over phenomena). Descriptive statistics: Categorical data was reported as number and percentages and continuous data as means and standard deviations (SD). Multilevel repeated measurements mixed effects model with participants as a random effects factor and the outcome variable as a dependent variable was used. The time (3 levels; month 0, 4, 12) was set as a fixed effect factor based on a restricted maximum likelihood model. While adjusting for baseline levels, the model facilitated comparisons between the time points and the trajectory over the entire study period. Inferential statistics is reported as least square means with standard errors and the differences between them with 95% confidence intervals (95%CI). All 95%CIs and P values are two sided. Safety was reported in a descriptive manner. A non-responder sensitivity analysis was performed with baseline observations carried forward to replace missing data. The statistical analyses were performed in SAS an R Studio.

Data availability

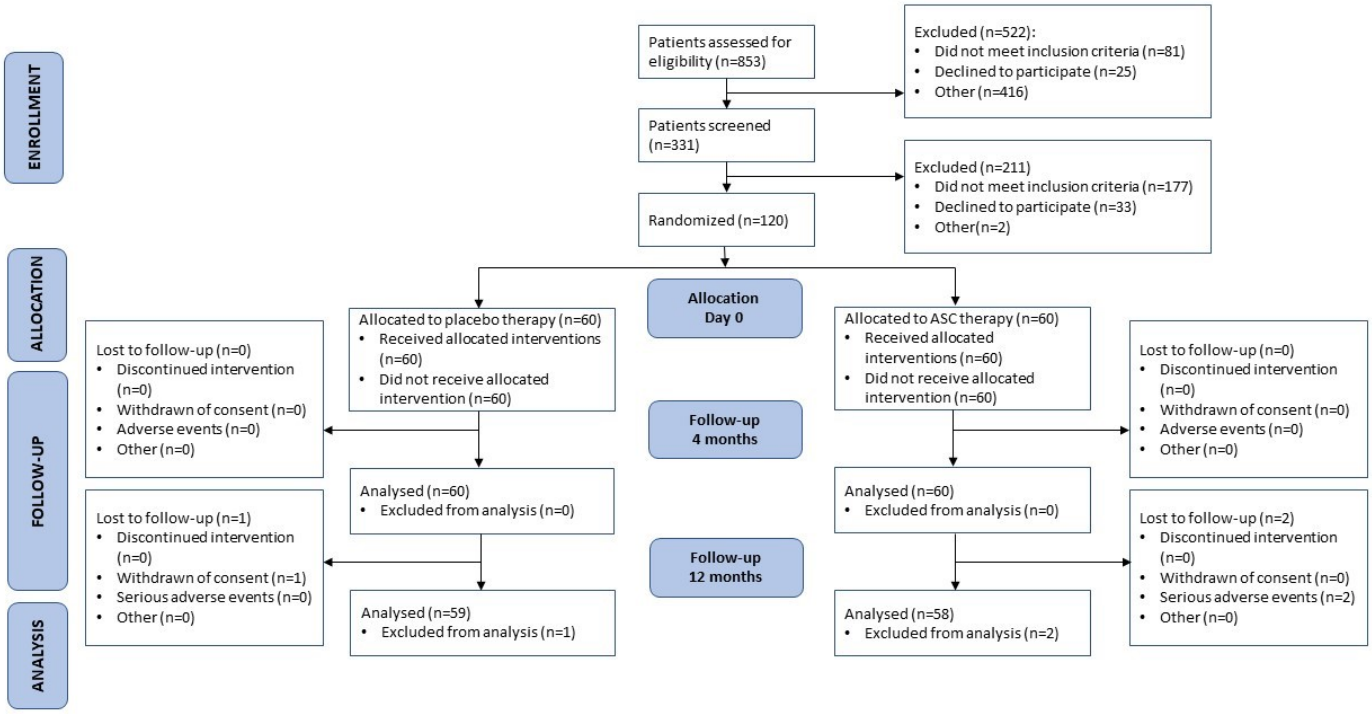
Due to sensitivity reasons, the data from this study is not publicly accessible. However, upon reasonable request to the corresponding author data are available.

Results

Study flow and baseline characteristics

A total of 120 patients with previous head and neck cancer were included at the Department of Otorhinolaryngology, Head and Neck Surgery & Audiology, Copenhagen University Hospital and received the intended intervention, while 117 were assessed at the 12-month follow-up visit. **Figure 1** illustrates the progress through the trial from enrolment to the 12-month follow-up (January 19th, 2021, to February 12th, 2024). Three patients were lost to follow-up: one patient (placebo) withdrew consent, and two patients (ASCs) were excluded due to new primary cancer in accordance with the protocol withdrawal criteria[26].

Figure 1. CONSORT Flow diagram.



The two groups were comparable at baseline[22]. Most patients had oropharyngeal cancer, 107 (89%), the mean age was 61.4 years (SD: 7.1 years), 88 (73%) were males, 89 (74%) had stage I-II, and were 53 (44%) never smokers. UWS at baseline was 0.13 mL/min (SD: 0.01 mL/min). See **Table 1.**

Table 1. Baseline characteristics in the intention-to-treat population.

Characteristics	**ASC** (n=60)	**Placebo** (n=60)
Age (years)	61.0 (7.4)	61.8 (6.8)
Male, sex, no. (%)	41 (68)	47 (78)
Performance score (0-1), no. (%)	60 (100)	60 (100)
Previous smoking history		
0 pack years	29 (48)	24 (40)
1-10 pack years	7 (12)	6 (10)
>10 pack years	24 (40)	30 (50)
Cancer location, no. (%)		
Oropharynx	52 (87)	55 (92)
Other	7 (12)	5 (8)
UICC8 stage, no. (%)		
I-II	45 (75)	44 (73)
III-IV	15 (25)	16 (27)
Mean radiation dose to the four large salivary gland, Gy	39.9 (7.1)	40.5 (8.9)
Salivary flow rate, mL/min		
Unstimulated whole saliva flow rate	0.13 (0.05)	0.13 (0.06)
Stimulated whole saliva flow rate	1.14 (0.59)	0.99 (0.60)
EORCT QLQ-H&N35 (0-100)		
HNDR	81.9 (23.4)	76.3 (23.2)
HNSS	55.6 (38.2)	57.1 (37.7)
HNSW	33.9 (22.8)	31.8 (19.9)
XQ (0-100)	51.0 (20.5)	47.9 (19.9)

Estimates are reported as means and SDs unless otherwise indicated.
Abbreviations: ASC, adipose-derived mesenchymal stem cells; EORCT QLQ-H&N35, The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire; Gy, gray; HNDR, domains for dry mouth; HNSS, domains for sticky saliva; HNSW, domains for swallowing; no., number; UICC, union for international cancer control; XQ, xerostomia questionnaire.

Salivary gland function

From baseline to 12 months, an increase in UWS of was observed in the ASC group (0.02 mL/min; 95% CI [0.01 to 0.04]) and in the placebo group (0.02 mL/min; 95% CI [0 to 0.03]), with no difference in change between the groups (0.01 mL/min; 95% CI [-0.02 to 0.03]), see **Table 2**. The UWS increased from 0.13 mL/min (95% CI 0.11 to 0.15) to 0.16 mL/min (95% CI 0.14 to 0.18) in the ASC group, and from 0.13 mL/min (95% CI 0.11 to 0.15) to 0.17 mL/min (95% CI 0.1 to 0.19) in the placebo group, see **Figure 2**. This

corresponded to an increase at 12 months of 25% (95% CI 18 to 34) in the ASC group and 27% (95% CI 20 to 33) in the placebo group compared to baseline. At 12 months, 23 ASC patients and 18 placebo patients had an increase in UWS >30% with 6 in the ASC group and 4 in the placebo group acquiring a normal saliva flow rate of $\geq$ 0.3 mL/min.

Table 2. Comparison of change from in primary outcomes from baseline to 12 months follow-up (trial secondary end point).

	ASC (n = 60)	**Placebo** (n = 60)	**Difference (95% CI)**	**p-value***
Change From Baseline to 12 months				
Unstimulated saliva flow rate, mL/min	0.02 (0.01)	0.02 (0.01)	0.01 (-0.02 to 0.03)	0.56
Stimulated saliva flow rate, mL/min	0.04 (0.03)	0.08 (0.03)	-0.04 (-0.17 to 0.04)	0.35
XQ-summary score (0-100)	-3.12 (1.18)	-2.74 (1.17)	-0.38 (-3.68 to 2.91)	0.82
EORCT QLQ-H&N35 (score 0-100):				
HNDR	-10.07 (1.68)	-4.15 (1.67)	-5.93 (-10.62 to -1.22)	0.01*
HNSS	-9.27 (2.17)	-4.55 (2.16)	-4.72 (-10.78 to 1.35)	0.13
HNSW	-4.50 (1.05)	-3.49 (1.05)	-1.00 (-3.94 to 1.94)	0.5

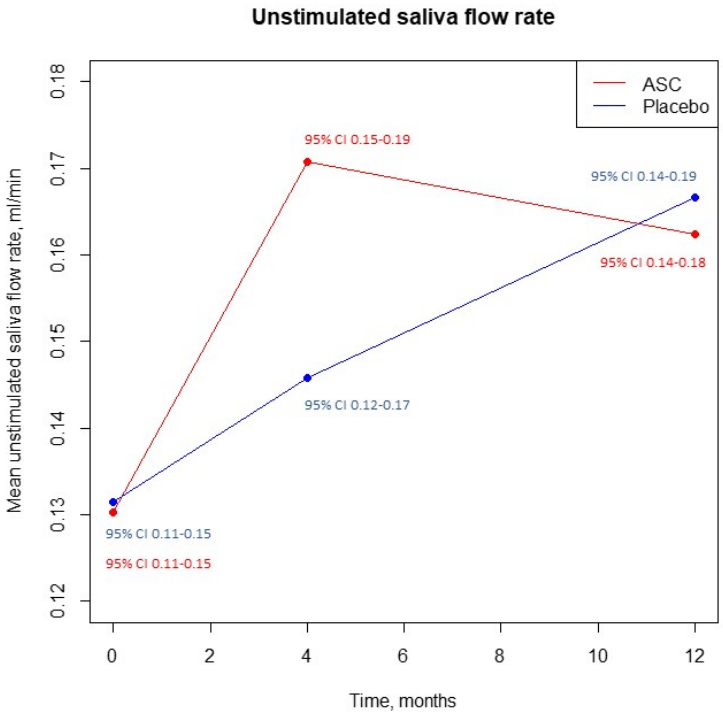
Values are least squares means with standard errors for each group; the difference is reported with 95% confidence intervals (95%CIs). Estimates are derived from the repeated measures, mixed effects models.

*Significant p-value.

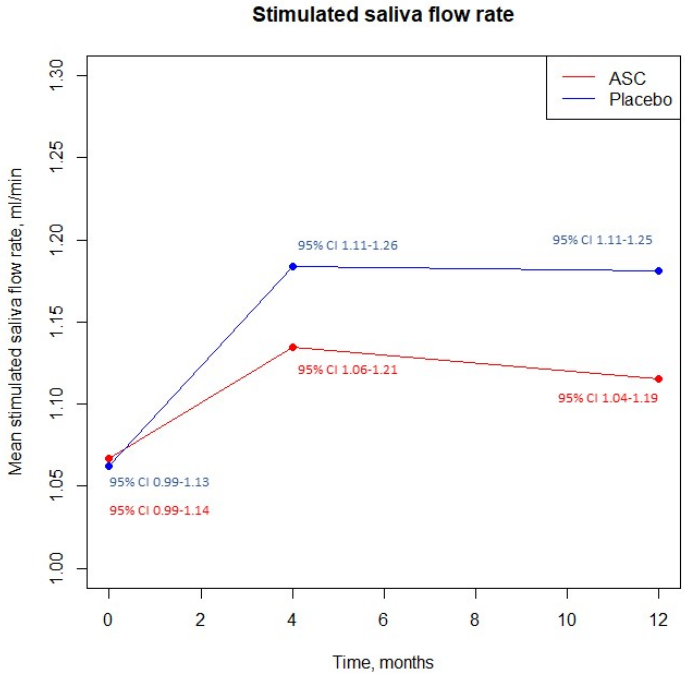
Abbreviations: ASC, adipose-derived mesenchymal stem cells; CI, confidence interval; EORCT QLQ-H&N35, The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire; HNDR, domains for dry mouth; HNSS, domains for sticky saliva; HNSW, domains for swallowing.

Figure 2. Change in salivary gland function measured over the 12 months study period. **A**. Unstimulated whole salivary flow rate (UWS) **B**. Stimulated whole saliva flow rate (SWS)

A.



B.



Abbreviations: ASC, adipose-derived mesenchymal stem cells; CI, confidence interval, min, minute; mL, milliliters.

From baseline to 12 months, an increase in SWS was observed in the ASC group (0.04 mL/min; 95% CI [-0.01 to 0.1]) and in the placebo group (0.08 mL/min; 95% CI [0.02 to 0.13]), with no difference in change between the groups (-0.04 mL/min; 95% CI [-0.12 to 0.04]), see **Table 2**. The SWS increased from 1.07 mL/min (95% CI 0.99 to 1.14) to 1.12 mL/min (95% CI 1.04 to 1.19) in the ASC group, and from 1.06 mL/min (95% CI 0.99 to 1.13) to 1.18 mL/min (95% CI 1.11 to 1.25) in the placebo group, see **Figure 2**. Overall, no difference between the ASC and placebo group was observed in salivary gland function.

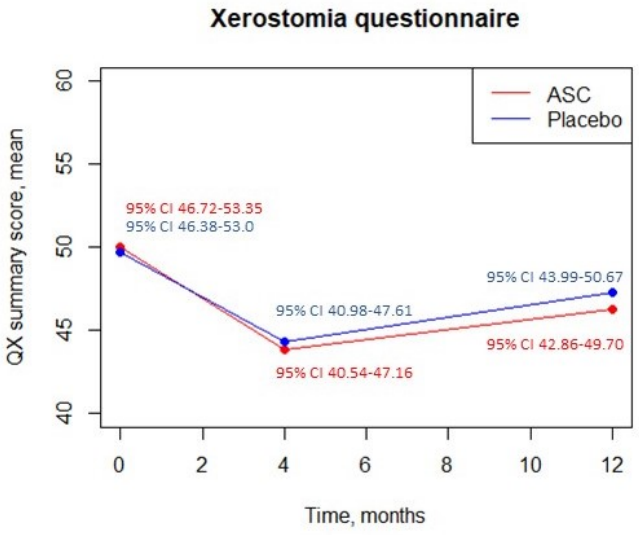
Three patients were lost to follow-up at the 12-month follow-up visit (one in the placebo group and two in the ASC group). No differences in mean salivary flow rates were observed in the non-responder analysis, see **Supplementary 2**.

Patient-reported outcome measurements

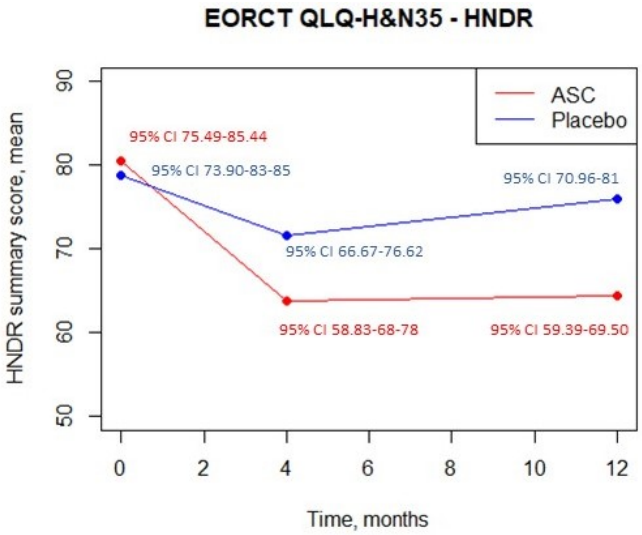
From baseline to 12 months, a decrease in EORCT-H&N35 HNDR sum score was observed in the ASC group (-10.07 units; 95% CI [-13.39 to -6.75]) and in the placebo group (-4.15 units; 95% CI [-7.46 to -0.84]), with a significant difference in change between the groups of -5.93 units (95% CI -10.62 to -1.22), see **Table 2**. The EORCT-H&N35 HNDR sum score decreased from 80.47 units (95% CI 75.49 to 85.44) to 64.45 units (95% CI 59.39 to 69.5) in the ASC group, and from 78.87 units (95% CI 73.90 to 83.85) to 75.97 units (95% CI 70.96 to 81) in the placebo group, see **Figure 3**. No difference in change between the ASC group and the placebo group was observed for EORCT-H&N35 domains HNSS (-4.72 units, [95% CI -10.78 to 1.35]) and HNSW (-1 unit [95% CI -3.94 to 1.94]), or XQ (-0.38 units [-3.68 to 2.91]).

Figure 3. Change in salivary gland function measured over the 12 months study period in the patient reported outcomes. **A**. Xerostomia questionnaire (XQ). **B**. EORCT-QLQ-H&N35 HNDR (domains for dry mouth). **C.** EORCT-QLQ-H&N35 HNSS (domains for sticky saliva) **D.** EORCT-QLQ-H&N35 HNSW (domains for swallowing).

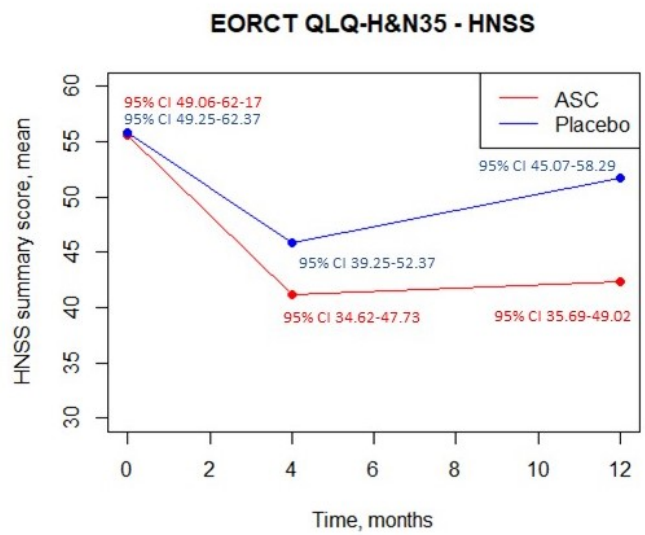
A.



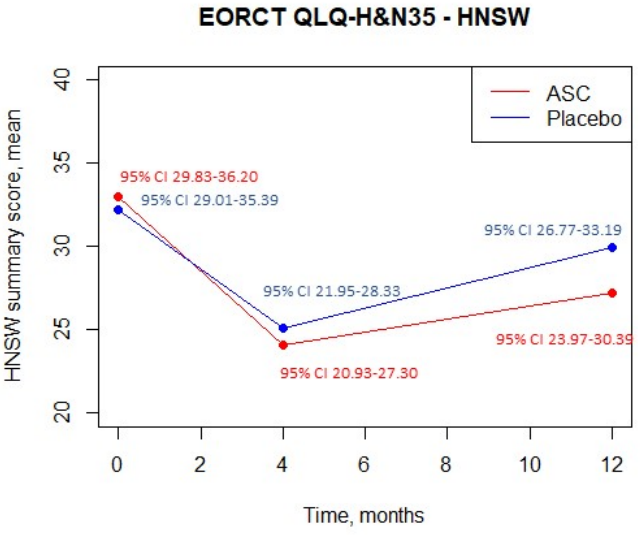
B.



C.



D.



Sum scores for both the XQ and the EORCT QLQ-H&N35 questionnaires range from 0-100. A Higher sum score is associated with increased symptom burden, while a lower sum score is associated with lower symptom burden.
Abbreviations: ASC, adipose-derived stem cells; CI, confidence interval; EORCT QLQ-H&N35, The European Organization for Research and Treatment of Cancer Quality of Life Questionnaire; Gy, gray; HNDR, domains for dry mouth; HNSS, domains for sticky saliva; HNSW, domains for swallowing; SMG, submandibular gland. XQ, xerostomia questionnaire.

Two patients (one in the ASC group and one in the placebo group) did not respond to all HNSW baseline questions, two patients did not respond to the XQ at 12 months (two in the ASC group) while three patients were lost to follow-up at 12 months (one the placebo group and two in the ASC group) and were consequently not included in the patient-reported outcomes analyses. No differences in mean scores were observed in the non-responder analysis, see **Supplementary 2**.

Safety and immune response

Through the 12 months study period no deaths were observed. In total, 16 SAEs were observed from baseline to the 12-month follow-up, and which were all deemed unrelated to the treatment. Three patients experienced a SAE from intervention to the 4-months follow-up (two in the placebo group and one in the ASC group), which are previously described[22]. From the 4-month to the 12-month follow-up additionally 13 SAEs occurred (six in the ASC group and seven in the placebo group). Four patients developed a new malignancy in the study period: two ASCs patients developed oesophageal cancer (one adenocarcinoma and one squamous cell carcinoma), one ASC patient developed both skin cancer and malignant melanoma, and one placebo patient developed chromophobe renal carcinoma. None of the SAEs were deemed related to the treatment and no difference was observed between ASCs and placebo, see **Table 3**. For further details see **Supplementary 3**.

Table 3. Safety profile up to 12 months from 4 months.

Adverse events	**ASC group (n=60)**	**Placebo group (n=60)**	**Risk Difference (95% CI)**
Deaths, no (%)	0 (0)	0 (0)	NA
Serious adverse events, no (%)	6 (10)	7 (12)	0.03 (-0.08-0.15)
Second primary cancer, no. (%)	4 (7)	1 (2)	
Infectious disease requiring hospitalization, no. (%)	0 (0)	3 (5)	
Other, no. (%)	2 (3)	3 (5)	
De novo donor specific HLA antibodies, no. (%)	23 (38)	NA	NA
Patients with persistent response from 4 to 12 m, no. (%)*	5 (8)		
Patients with increased response from 4 to 12 m, no. (%)*	1 (2)		
Patients with reduced response from 4 to 12 m, no. (%)*	8 (13)		
Patients with resolved response from 4 to 12 m, no. (%)**	8 (13)		
Unknown status at 12 months, no. (%)	1 (2)		

*****An increase or decrease is defined as a change in mean florescence intensity (MFI) sum of donor specific antibodies $\geq$ 3000. **Resolved response is defined as no detection of donor specific antibodies in a patient with a former de novo response.

In all, 23 (38%) patients receiving ASCs experienced an immune response to the treatment and developed de novo HLA class I donor specific antibodies. Of these, 8 (35%) patients experienced a resolved immune response at 12 months follow-up with no detectable donor specific antibodies remaining. Eight (35%) patients had a reduction in the immune response, 5 (22%) patients had a persistent immune response, and one patient (4%) had an increased immune response. One patient (4%) was lost to follow-up. See **Table 3**. For further details see **Supplementary 4**.

Subgroup analysis

Higher mean radiation dose to the four major salivary glands was associated with less increase in UWS with an average -0.004 mL/min (95% CI -0.01 to 0) per increase in Gy. Also, patients who developed donor specific antibodies (-0.01 mL/min [95% CI -0.07 to 0.05]), age over 60 (-0.04 mL/min, [95% CI -0.09 to 0.01]) and previous smokers (-0.03 mL/min [95% CI -0.07 to 0.02]) tended to experience a lower increase in UWS.

Discussion

This study represents the long-term results on effectiveness and safety of the MESRIX-III clinical trial: the largest human randomised trial investigating ASCs for radiation-induced salivary gland hypofunction and xerostomia in head and neck cancer survivors. At 12 months following intervention, we found an increase in UWS in both arms, with a 25% increase in the ASC group and a 27% increase in the placebo group, with no statistically significant difference between the two groups (difference in change -0.01 mL/min [95% CI -0.02 to 0.03]).

We found a significant decrease in the symptom burden related to dry mouth (EORTH-H&N35 HNDR) for ASC treated patients from 80.47 units (95% CI 75.49 to 85.44) to 64.45 units (95% CI 59.39 to 69.5) compared to a placebo (from 78.87 units; 95% CI [73.90 to 83.85] to 75.97 units; 95% CI [70.96 to 81]) favouring ASC therapy at 12 months. Significant long-term effect of ASCs compared to placebo on patients-reported outcomes was also observed in the MESRIX-I study[24]. In addition, we found a decrease in the symptom burden for all other patient-reported outcomes including sticky saliva, swallowing, and xerostomia, with no significant differences in change between the groups (HNSS -4.72 units; 95% CI [-10.78 to 1.35, HNSW -1 unit; 95% CI [-3.94 to 1.94], XQ -0.38 units; 95% CI [-3.68 to 2.91]). However, the reduction in sum scores for all patient-reported outcomes was most pronounced in the ASC group and approached a potentially clinically significant change of 5-10 units, as indicated by previous studies[29,30].

The increase in both salivary gland function and patient-reported outcomes observed in the placebo group is noteworthy. Improvements in salivary gland function and xerostomia related symptoms is not expected more than two years after radiotherapy[31,32], although a continuous recovery have been observed up to four years post-RT[32]. These results indicate a continuous recovery following radiation-induced salivary gland damage. However, it could also indicate that the placebo Cryostor10 (BiolifeSolutions) containing 10% DMSO may act as a therapeutic agent in salivary gland damage, and DMSO may be anti-inflammatory as shown in other diseases[33–35].

We did not observe any differences between the groups in long-term effect on SWS, with a difference in change of -0.04 mL/min (95% CI -0.17 to 0.04), corresponding to a 10% increase in the ASC group and a 11% increase in the placebo group. We did not treat the parotid glands, which are responsible for 50% of the stimulated saliva secretion[36], thus we did not expect changes in the SWS, and the effect was also lower than the effect on UWS. This supports the indication of a continuing natural salivary gland repair following RT.

No treatment-related SAEs following ASC therapy were observed, and we did not observe a risk difference between ASCs and placebo. This is in line with our previous MESRIX-studies[20,21,24,25] and others[23]. Four patients developed a new malignancy in the study period (three in the ASC group and one in the placebo group). There is no indication that MSC therapy leads to malignancy or undergo malignant transformation *in vivo*[17,37,38]. Instead, previous cancer patients, including head and neck cancer patients, are at risk of developing a new secondary primary cancer[39,40]. Also, patients were older (mean age 61.4 years) and with a history of smoking (56%) which also increases the risk of cancer.

In total, 38% of ASC patients developed HLA class I donor specific antibodies. Most of these either had a reduced or a resolved response at 12 months compared to 4 months, indicating a transient immune response, which is also described for patients receiving platelet transfusions[41]. Subgroup analysis revealed a tendency to a less increase in UWS in those who developed donor specific antibodies. Immunization with

HLA antibodies is known to cause challenges in solid organ transplantation and may eventually be important for a very small subset of patients receiving treatment with ASCs[42].

In line with the results from the MESRIX-III at 4 months follow-up[22], exploratory subgroup analyses also revealed that high mean radiation dose to the four major salivary glands was associated with a less increase in UWS (-0.004 mL/min, 95% CI [-0.01-0.00] per increase in Gy). Also, age over 60 years, and ever smokers trended to experience a lower effect of ASCs on UWS. Moving forward, further investigation is warranted to comprehensively elucidate the nuanced interplay between ASC therapy and patient-specific factors influencing treatment outcomes. Identifying potential subgroups might contribute to refining therapeutic strategies and optimizing patient care. This could also involve ASC treatment immediately following RT, when the salivary glands have a higher potential for regeneration, unlike the two-year post-treatment inclusion criteria which was used in this study.

While the observed increase in salivary flow rates suggests a degree of functional improvement, the absence of a significant difference between ASCs and placebo underscores the intricacies involved in restoring salivary gland function post-RT and prompt a deeper investigation into the mechanisms underlying salivary gland regeneration and the effect of ASC therapy. In addition, multiple administrations with MSCs might promote sustained beneficial effects compared to a single administration in both a variety of neurological diseases and in osteoarthritis[43–47]. Similarly, repeated treatment with ASCs may hold a potential to further improve saliva production in radiation-induced xerostomia, but has currently not been investigated.

This study was limited by the unblinded design at 12 months with both participants and study personnel being unblinded to the intervention, in contrast to the follow-up at 4 months which was blinded. This might overestimate the effect of ASCs while also underestimating the effect of placebo. Although this study is the largest human clinical trial investigating intraglandular ASCs for radiation-induced xerostomia, it remains insufficient for detailed exploration of potential subgroups, that may experience a more pronounced benefit from ASC therapy. Larger, multicentre studies are needed to further investigate if patient-specific variables impact treatment outcomes.

In conclusion, this study showed the first long-term results of a randomised, placebo-controlled phase II clinical trial investigating intraglandular ACS therapy for radiation-induced xerostomia and hyposalivation in head and neck cancer survivors. ASC therapy demonstrated a significant improvement in alleviating the subjective feeling of dry mouth compared to placebo at 12 months. While significant increases in unstimulated flow rates were observed for both ASCs and placebo, our findings did not establish the superiority of ACSs over placebo to restore salivary gland function as assessed by objective salivary flow rate measurements.

Author's contributions

ALFC: Conceptualization, resources, data curation, funding acquisition, formal analysis, validation, investigation, methodology, validation, writing–original draft, project administration. **KKJ**: Conceptualization, resources, data curation, funding acquisition, validation, investigation, methodology, writing–review and editing, project administration. **TT**: Supervision, validation, methodology, writing – review and editing. **NP**: Data curation, methodology, project administration, writing – review and editing. **AKØM**: Data curation, methodology, writing – review and editing. **SKB**: Data curation, writing – review and editing. **JK:** Conceptualization, supervision, validation, methodology, writing – review and editing. **AE**: Conceptualization, writing – review and editing. **MHS**: Conceptualization, writing – review and editing. **MF** Data curation, writing – review and editing. **CM**: Data curation, writing – review and editing. **JF**: Data curation, supervision, writing – review and editing. **CDL**: Conceptualization, methodology, writing – review and editing. **AWH**: Data curation, writing – review and editing. **RC** Formal analysis, supervision, writing – review and editing. **CG**: Conceptualization, resources, supervision, funding acquisition, validation, investigation, methodology, writing – review and editing. **CvB** Conceptualization, resources, supervision, funding acquisition, validation, investigation, methodology, writing – review and editing.

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Supplementary 1. **Deviations to protocol from month 4 to month 12.**

Deviation	Number of patients	Reason	Consequence
Follow-up time was too late (more than 12 months +/- 4 weeks)	1	Due to illness, work commitments, and others, one patient rescheduled their appointment.	Registered in the trial master file.
Received a smaller amount of paraffin wax during sialometry at 12 months measuring stimulated salivary flow rate	8 (4 ASC and 4 placebo)	Measuring error at baseline.	Registered in the trial master file. An equivalent amount was used for the subsequent follow-up visits.
Sialometry was not conducted simultaneously at the same time of day at 12 months as the previous assessments.	1	Due to work commitments and other, one patient rescheduled their appointment	Registered in the trial master file.

Supplementary 2. Sensitivity analysis. Non-responder imputation with baseline values carried forward.

	ASC (n = 60)	**Placebo** (n = 60)	**Difference (95% CI)**	**p-value***
Change From Baseline to 12 months				
Unstimulated saliva flow rate, mL/min	0.02 (0.01)	0.02 (0.01)	0.01 (-0.02 to 0.03)	0.57
Stimulated saliva flow rate, mL/min	0.04 (0.03)	0.08 (0.03)	-0.04 (-0.11 to 0.04)	0.36
XQ-summary score (0-100)	-3.25 (1.15)	-2.63 (1.15)	-0.62 (-3.84 to 2.60)	0.70
EORCT QLQ-H&N35 (score 0-100):				
HNDR	-9.77 (1.67)	-4.12 (1.67)	-5.65 (-10.35 to -0.95)	0.02*
HNSS	-8.99 (2.18)	-3.98 (2.18)	-5.01 (-11.11 to 1.09)	0.11
HNSW	-4.82 (1.05)	-2.86 (1.05)	-1.96 (-4.91 to 0.99)	0.19

Abbreviation: EORTC QLQ-H&N35: the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire, Head and Neck Module; HNDR: domains for dry mouth (HNDR); HNSS: domains for sticky saliva; HNSW: domains for swallowing; XQ: xerostomia Questionnaire; ASC: adipose-derived mesenchymal stem/stromal cells

Supplementary 3. Serious adverse events from month 4 to month 12.

In total, 13 patients had a serious adverse event (SAE) from the 4-month follow-up to the 12-month follow-up. Of these, seven patients received placebo and six patients received adipose-derived mesenchymal stromal cells.

Patients who received placebo experienced: One patient were diagnosed with chromofobe renal cell carcinoma (stage pT1a) and underwent partial nefrectomy. The patient was deemed curative treated; one patient had pneumonia and was hospitalized to receive i.v antibiotics for 4 days; one patient had pyelonephritis and were hospitalized to receive i.v antibiotics for one day; one patient had gallstones complicated with cholecystitis and was cholecytectomised; one patient was admitted for insertion of a canalicular monostent due to persistent tearing; one patient underwent gastroscopy to due epigastric pain and was diagnosed with inflammation; one patient were diagnosed with a laryngeal papilloma without suspicion of cancer which was surgically removed.

Patients who received ASCs experienced: One patient were diagnosed with esophageal cancer (stage T3N1M0) five months following the intervention and received palliative treatment; one patient had skin squamous cell carcinoma which was surgically removed and the patient was deemed curatively treated. The patient had a history of both skin squamous cell carcinoma and malignant melanoma. The same patients were also diagnosed with malignant melanoma at the 4-month follow-up as reported previsouly[1]. One patient had complicated kidney stones and was hospitalized to endoscopic removal; one patient was hospitalized due to amaurosis fugax and no underlying diagnosis was established; one patient was diagnosed with esophageal cancer (stage cT4bN2M0) seven months following the intervention and received palliative treatment. None of the SAEs were deemed related to the treatment. The two patients who experienced esophageal cancer were lost to follow-up, since they received palliative treatment.

Supplementary 4. Immune response and development of HLA antibodies from baseline to 12 months in the ASC group.

Donor 1: HLA-A3,-; B18,37; Cw6,7; DR4,14; DR52, DR53; DQ5(1),8(3); DPB1*02:01,16:01

Donor 2: HLA-A2,11; B57,B62(15); Cw9(3),6; DR7,13(6); DR52,DR53N; DQ6(1),9(3); DPB1*04:01,-

Donor 3: HLA-A2,3;B7,-;Cw7,-;DR15(2),-;DR51;DQ6(1),-; DPB1*02:01,04:01.

Trial ID	Donor	Donor ID	Donor-specific HLA DSA (MFI)				Antibody types detected
			HLA Antigen	Baseline MFI	4 months MFI	12 months MFI	
002	1	IH23	A3 B18 B37 DQ5	(-) 4000 2000 4000	3000 10000 5000 4000	(-) 6000 3000 5000	De novo DSA and preformed DSA
003	1	IH23	-	(-)	(-)	(-)	
007	1	IH23	-	(-)	(-)	(-)	
008	1	IH23	B37	2000	1000	2000	Preformed DSA
010	1	IH23	-	(-)	(-)	(-)	
015	2	AA25	B57 B62	(-) (-)	2000 2000	1000 (-)	De novo DSA
016	2	AA25	-	(-)	(-)	(-)	
018	2	AA25	A11	(-)	(-)	4000	
020	1	IH23	-	(-)	(-)	(-)	
021	2	AA25	A2 B57 B62(15)	(-) (-) (-)	2000 6000 1000	1000 5000 2000	De novo DSA
022	2	AA25	-	ND	(-)	(-)	
024	2	AA25	A2 B57 B62(15) Cw9(3)	(-) (-) (-) (-)	7000 14.000 2000 1000	5000 6000 1000 (-)	De novo DSA
026	2	AA25	B57 B62(15)	(-)	2000 1000	(-) (-)	De novo DSA
028	1	IH23	-	(-)	(-)	(-)	
029	1	IH23	-	(-)	(-)	(-)	
033	1	IH23	-	(-)	(-)	(-)	
034	1	IH23	-	(-)	(-)	(-)	
035	1	IH23	-	(-)	(-)	(-)	
037	3	ML28	A2 A3 B7	(-) (-) (-)	16000 7000 15000	ND ND ND	De novo DSA
038	3	ML28	-	(-)	(-)	(-)	
040	3	ML28	B7	(-)	1000	2000	De novo DSA
041	3	ML28	-	(-)	(-)	(-)	
043	3	ML28	A3 DQ6	(-) 3000	2000 3000	(-) 3000	De novo DSA
047	3	ML28	(-)	(-)	(-)	(-)	
049	3	ML28	A3 B7 DR15 DR51 DQ6(1)	(-) 3000 10000 11000 6000	10000 22000 10000 11000 6000	9000 23000 9000 12000 6000	De novo DSA and preformed DSA
052	3	ML28	-	(-)	(-)	(-)	
053	3	ML28	-	(-)	(-)	(-)	
057	3	ML28	A2	(-)	<2000	(-)	De novo DSA
059	3	ML28	A3 B7 DQ6(1)	(-) (-) 7000	12000 8000 7000	9000 5000 7000	De novo DSA

060	2	AA25	-	(-)	(-)	(-)	
061	3	ML28	-	(-)	(-)	(-)	
064	3	ML28	-	(-)	(-)	(-)	
066	2	AA25	B62(15) Cw9	2000 2000	12000 9000	9000 8000	Preformed DSA
069	3	ML28	-	(-)	(-)	(-)	
071	3	ML28	-	(-)	(-)	(-)	
072	3	ML28	-	(-)	(-)	(-)	
073	2	AA25	A2 B57 B62(15) DQ6(1)	(-) (-) (-) 1000	2000 5000 1000 1000	(-) (-) (-) (-)	De novo DSA
074	2	AA25		(-)	(-)	(-)	
078	3	ML28	-	(-)	(-)	(-)	
080	2	AA25	A2 A11 B57 B62(15)	(-) (-) (-) (-)	7000 2000 14000 5000	2000 (-) 3000 3000	De novo DSA
081	3	ML28	-	(-)	(-)	(-)	
084	3	ML28	A3 B7	(-) (-)	1000 2000	(-) (-)	De novo DSA
086	2	AA25	-	(-)	(-)	ND	
087	3	ML28	A2	(-)	2000	(-)	De novo DSA
089	1	IH23	-	(-)	(-)	(-)	
090	3	ML28	A2 A3	(-) (-)	8000 6000	1000 (-)	De novo DSA
093	3	ML28	-	(-)	(-)	(-)	
094	1	IH23	-	(-)	(-)	(-)	
098	1	IH23	-	(-)	(-)	(-)	
099	3	ML28	-	(-)	(-)	(-)	
103	3	ML28	-	(-)	(-)	(-)	
104	3	ML28	-	(-)	(-)	(-)	
105	3	ML28	A2 A3 B7	(-) (-) (-)	6000 2000 4000	(-) (-) 2000	De novo DSA
108	1	IH23	-	(-)	(-)	(-)	
109	1	IH23	-	(-)	(-)	(-)	
110	2	AA25	A2 B57	(-) (-)	2000 2000	(-) (-)	De novo DSA
114	3	ML28	-	(-)	(-)	(-)	
117	1	IH23	Cw7	1000	(-)	(-)	Preformed DSA
119	3	ML28	A2 A3 B7	(-) (-) (-)	5000 7000 5000	(-) 1000 (-)	De novo DSA
120	1	IH23	-	(-)	(-)	(-)	

Abbreviations: DSA: Donor specific antibodies; HLA: human leucocyte antigen; ID: identification; MFI: normalized mean fluorescence intensity; ND = not determined; (-): negative.
MFI is approximate values (rounded to the nearest thousand). Negative is defined as MFI < 1000 for Labscren Single Antigen, One Lambda, NBG<3.0 for HLA class I and NBG<4.0 for HLA class II for Labscreen Mixed, One Lambda

Supplementary 5. Statistical Analysis Plan

Statistical Analysis Plan

Title

Long-term Effectiveness and Safety of Mesenchymal Stromal Cell Therapy for Alleviating Radiation-Induced Hyposalivation and Xerostomia in Head and Neck Cancer Survivors: Statistical Analysis Plan for the secondary analyses of the Single-center, Double-blind, Randomized, Placebo-Controlled MESRIX-III Trial

Trial Registration

Danish Data Protection Agency (protocol number P-2020-1164)

The National Ethics Committee protocol number: (Protocol number: 1802872)

The Danish Medical Agency (2018-000348-24)

ClinicalTrials.gov database (NCT04776538)

SAP version:

Version 1, 3st of March 2024

SAP associated with protocol version 2.10

Primary authors of the SAP: Amanda-Louise Fenger Carlander, Kathrine Kronberg Jakobsen and Robin Christensen

Roles and responsibilities

Senior biostatistician responsible: Robin Christensen, BSc, MSc, PhD, Professor

Principal investigator: Kathrine Kronberg Jakobsen, MD, PhD-fellow

Investigator: Amanda-Louise Fenger Carlander, MD, PhD-fellow

Sponsor: Christian von Buchwald, MD, DMSc, Professor

This SAP is reported following the recommendations from "Guidelines for the Content of Statistical Analysis Plans in Clinical Trials." by Gamble C, Krishan A, Stocken D, Lewis S, Juszczak E, Doré C, et al. published in JAMA 2017;318:2337-43.

Indholdsfortegnelse

Introduction

Background and rationale

A prevalent adverse outcome of radiotherapy in head and neck cancer cases is the diminished function of salivary glands leading to xerostomia. Intraglandular therapy involving mesenchymal stem cells has exhibited promising outcomes in addressing xerostomia[1–4]. This trial aims to assess the long-term effectiveness and safety of administering adipose-derived mesenchymal stem cells (ASC) through submandibular gland injection as a potential novel, disease-modifying intervention for post-radiation xerostomia. Anticipated results could establish a foundation for a clinically feasible approach to alleviate xerostomia in head and neck cancer survivors previously treated with radiotherapy.

Objectives

Primary objective

To compare the effect of ASC injection, relative to placebo, on changes in unstimulated salivary gland function from baseline to month 12:

i.e., effectiveness: change in salivary gland function is measured by 12 months change in unstimulated whole saliva flow rate between ASC and placebo.

Secondary objectives

To compare the effect of ASC injection, relative to placebo, from baseline to month 12 on the following outcomes:

1. Effectiveness: Changes in stimulated salivary gland function measured by 12 months change in stimulated whole saliva flow rate.
2. Patient-reported outcome: The European organization for research and treatment of cancer quality of life questionnaire, head and neck-35 (EORTC QLQ-H& N35):
    - Change in domains for dry mouth (HNDR)
    - Change in domains for sticky saliva (HNSS)
    - Change in domains for swallowing (HNSW)
3. Patient-reported outcome: Xerostomia Questionnaire (XQ).

Safety objectives

1. Safety up to 12 months duration from baseline will be evaluated by the incidence of:

a) Serious adverse events
b) Deaths

2. Immunological response to treatment: up to 12 months duration from baseline, evaluated by the

a) Development of de novo HLA antibodies as a response to ASC treatment in the first 12 months of the study period.
    - Patients with persistent antibodies from 4 months to 12 months
    - Patients with increased antibodies from 4 months to 12 months
    - Patients with reduced antibodies from 4 months to 12 months
    - Patients with resolved antibodies from 4 months to 12 months

Study methods

Trial design

The study was a randomized, single-center, double-blinded, placebo-controlled trial to compare the safety, tolerability, and effectiveness of intraglandular allogeneic ASCs as a treatment for radiation-induced hyposalivation and xerostomia in previous head and neck cancer patients. We intended to treat 120 patients with xerostomia, randomized in a 1:1 ratio, to receive ultrasound-guided injections of either ASCs or placebo in the submandibular glands. Placebo consisted of CryoStor10 (BiolifeSolutions), the freeze media for ASCs containing 10% Dimethyl sulfoxide (DMSO). Patients were followed up after four month (+/- 14 days) and after 12 months (+/- 4 weeks). The trial design is previously described[5].

Randomization

A predefined randomization code was established for all 120 patients according to patient treatment order (1-120) from the start of the trial. The allocation sequence was generated using www.randomization.com. The table with randomization numbers was available to one specified person at the Cardiology Stem Cell Centre (CSCC), The Heart Centre, Rigshospitalet, who was not involved in analyzing data related to the study endpoints. Randomization was performed in blocks of six, three patients receiving ASCs and three patients receiving placebo. Within blocks of six a balanced use of donors was applied. For the purpose of safety and potential unblinding, two sets of sealed envelopes containing the randomization code for each patient were made. Envelopes were locked up at CSCC production facility until allocation of treatment. After treatment one envelope remained at the CSCC and one envelope was kept at the clinical site at sponsor and thereby available for the investigator. The randomization is previously described[5].

Blinding

The sponsor, investigators, study staff (except for staff involved in stem cell preparation and staff involved in bioanalytical analyses), and patients were blinded to treatment assignment. A project nurse thawed the frozen suspension of ASCs or placebo before injection, and the syringes were covered in sterile green tape to ensure that neither the patients nor the study staff could see the suspension injected. After a follow-up period of 4 months, both patients and study staff were informed about the treatment, resulting in an unblinded 12-month follow-up phase. The blinding is previously described[5].

Sample size and power considerations

From our previous study MESRIX-I we assessed the increase in saliva production for whole unstimulated saliva flow rate (in ml/min) by about 33% or in absolute numbers from 0.125 to 0.155 after 4 months. The

power calculation was derived from a power of 0.8 and an alpha of 0.05. Consequently, the required total number of patients for inclusion would be 100, with 50 allocated to each group. We expected a dropout rate of up to 20% and therefore aimed to include 120 participants in total (i.e., representing the intention-to-treat population). The sample size and power consideration are previously described[5].

Framework

The trial was designed as a superiority trial, with the primary objective of showing that the response to the investigational product was superior to the comparative agent (experimental intervention and placebo comparator, respectively) as previously described[5].

Statistical interim analyses and stopping guidance

Four months data has been published[4].

Timing of final analysis

The primary endpoint, as well as the key secondary outcomes will all be evaluated based on the 12 months assessment.

Timing of outcome assessments

Table 1. Schedule of enrolment, interventions, and assessments.

	Study period			
	Enrolment	**Allocation**	**Post-allocation**	**Close-out**
Timepoint	Up to 90 days prior to intervention	0	4 months after intervention	12 months after intervention
ENROLMENT:				
Eligibility screen	X			
Informed consent	X			
Allocation		X		
INTERVENTION:				
ASC		X		
Placebo		X		
ASSESSMENTS:				
Saliva flow rate	X		X*	X
Saliva quality	X		X**	
QoL questionnaires	X		X*	X
HLA-response	X		X*	X
Safety			X*	X
Ultrasound of the submandibular glands	X		X*	X

*Reported in primary paper[4].

**Reported in separate paper.

Statistical principles

Confidence intervals and P values

All 95% confidence intervals and P values will be two sided. We will not apply explicit adjustments for multiplicity, rather we will analyze the key secondary outcomes in a prioritized order (e.g., "gatekeeping procedure"): The analyses of the key secondary outcomes will be performed and interpreted in sequence until one of the analyses potentially fails to show the statistically significant difference, or until all analyses have been completed at a statistical significance level of 0.05. The key secondary statistical tests will be reported with P values for hypothesis tests and claims of statistical significance.

Adherence and protocol deviations

Adherence is defined as participants who has a full registration and has completed every assessment for all the time points in the study. The adherence to the assessments will be summarized with number and percent compliance. All deviations from the protocol will be described.

Analysis populations

The primary analyses will be based on the Intention to Treat (ITT) population, i.e., based on the Full Analysis Set. The ITT principle asserts the effect of a treatment policy (that is, the planned treatment regimen), rather than the actual treatment given (i.e., it is independent of treatment adherence). Accordingly, participants allocated to a treatment group (X_{ASC} and $X_{Placebo}$, respectively) will be followed up, assessed, and analyzed as members of that group, irrespective of their adherence to the planned course of treatment (i.e., independent of withdrawals and cross-over phenomenon).

We will use a (multilevel) repeated measures mixed effects model with participants as a random effects factor and the particular outcome variable (Yi) as a dependent variable. The time (months; 3 levels: 0, 4, 12) is set as a fixed effect factor based on a restricted maximum likelihood model. While also adjusting for the level at baseline, the statistical model holds all between-time comparisons for assessment points up to 12 months from baseline (day 0), and allows for evaluation of the changes, as well as the trajectory over time from baseline to 12 months follow-up.

Trial population

Screening data, eligibility, and recruitment

Information on screening, eligibility, recruitment and withdrawal was published in the primary MESRIX-III paper[4].

Baseline patient characteristics

Descriptive statistics for categorical data will be evaluated as numbers and percentages while continuous data will be summarized by mean and standard deviation. We will not perform tests of statistical

significance for baseline characteristics. Baseline patient characteristics was published in the primary MESRIX-III paper[4].

Analysis

Outcome definitions

1. Effectiveness: Saliva gland function measured as the change in unstimulated whole saliva flow rate in the group receiving ASCs compared with the group of participants receiving placebo. Timeframe: 12 months. The saliva flow rate will be measured as ml/min.
2. Effectiveness: Saliva gland function measured as change in stimulated saliva flow rate in the group receiving ASCs compared with the group of participants receiving placebo. Timeframe: 12 months. The saliva flow rate will be measured as ml/min.
3. Effectiveness: Impact on quality of life measured as a change in patient-reported outcome of quality of life and xerostomia. Timeframe: 12 months. Patients will fill out the EORTC QLQ-H&N35.
    a. HNDR
    b. HNSS
    c. HNSW
4. Effectiveness: Impact on quality of life measured as a change in patient-reported outcome of quality of life and xerostomia. Patients will fill out the XQ. The results will be reported as a collected score. Timeframe: 12 months
5. Safety: evaluated by the number of patients with serious adverse events. Timeframe: 12 months.
    a. Development of serious adverse events
    b. Deaths
6. Immune response to treatment with ASC: Development of HLA antibodies as a response to ASC from 4 months to 12 months (de novo donor specific HLA antibodies) measured as sum mean fluorescence intensity (MFI) sum.
        a. Patients with persistent MFI sum from 4 months to 12 months
        b. Patients with increased MFI sum $\geq$ 3000 from 4 months to 12 months
        c. Patients with reduced MFI sum $\geq$ 3000 from 4 months to 12 months
        d. Patients with resolved MFI sum from 4 months to 12 months

Analysis methods

The repeated measures designs aim to draw conclusions about the mean values of the populations from which participants are selected by considering treatment and time effects in the model. The objective is usually achieved by considering both treatment and time effects (as well as the interaction between them) in the model. Data will be analyzed using SAS or Rstudio, with the particular outcome variable (Yi) as the dependent variable, using a multilevel repeated measures random effects model with participants as the random effect factor, and time (with 3 levels, incl. baseline) as fixed effect factors based on a restricted maximum likelihood (REML) model. This statistical model will hold all between-time comparisons for all assessment points up 12 months from baseline (incl. baseline) and thus allows for evaluation of the average effect, as well as the trajectory over time from baseline to 12 months follow-up.

Sensitivity

Exploring the robustness of the main analyses is a concept that refers to the sensitivity of the overall conclusions to various limitations of the data, assumptions, and analytic approaches to data analysis. Robustness implies that the treatment effect and primary conclusions of the trial are not substantially affected when analyses are carried out based on alternative assumptions or analytic approaches.

Loss to follow-up and missing data for various reasons is difficult to avoid in randomized trials and in pragmatic trials. We will apply the analysis framework suggested by White et al (2011) in which missing data related to the ITT approach depend on making plausible assumptions about the missingness of the data and including all participants in subsequent sensitivity analyses:

1. Attempt to follow up all randomized participants, even if they withdraw from allocated treatment (i.e., contact all individuals unless they explicitly stated that they had withdrawn their consent)
2. Perform a main analysis of all observed data that are valid under a plausible assumption about the missingness of the data (i.e., using Multiple imputation, assuming that data are 'Missing at Random' [MAR])
3. Perform sensitivity analyses to explore the effect of departures from the assumption made in the main (#2) analysis (i.e., a non-responder-imputation: using the value at baseline to replace missing data will correspond to a non-responder imputation; these models will potentially be informative even if data are 'Missing Not At Random' [MNAR])
4. Account for all randomized participants, at least in the sensitivity analyses (covered by #2 and #3 above, plus the corresponding analyses based on the per protocol population).

The interpretation of the corresponding statistical measures of uncertainty of the treatment effect and treatment comparisons will involve consideration of the potential contribution of bias to the P-value, 95% confidence interval, and of the inference in general.

Ad#1+2: Our primary analysis population will be all participants with available data at baseline, statistically modelled using repeated-measures linear mixed models (see above). These models will be valid if data are 'MAR'.

Ad#3+4 Sensitivity: We will analyze all variables, with missing data being handled by a simplistic non-responder imputation techniques (i.e., baseline observation carried forward).

When the different sensitivity analyses agree, and the analyses on the sensitivity analyses and the main analysis leads to essentially the same conclusions, confidence in the trial results is increased.

Harms

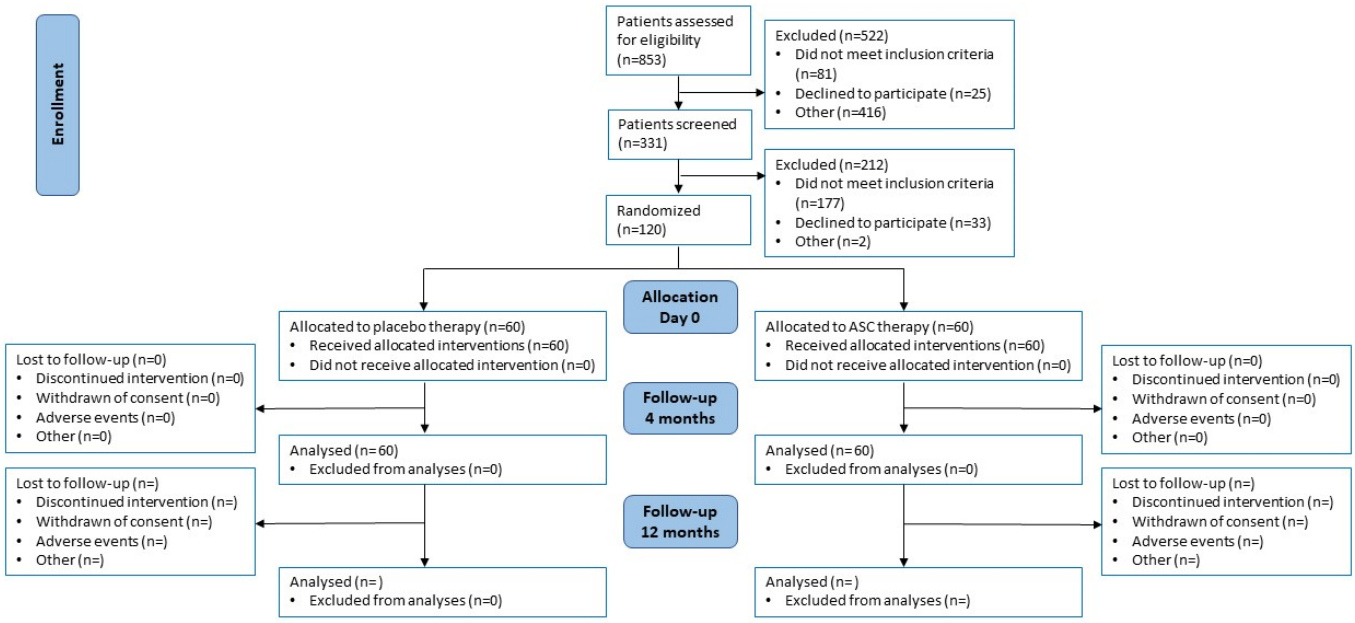
Any serious adverse events during the trial will be noted and reported in Table 3.

Statistical software

All statistical analyses will be performed in SAS and R-studio.

MANUSCRIPT OUTLINE *(Mock Ups)*

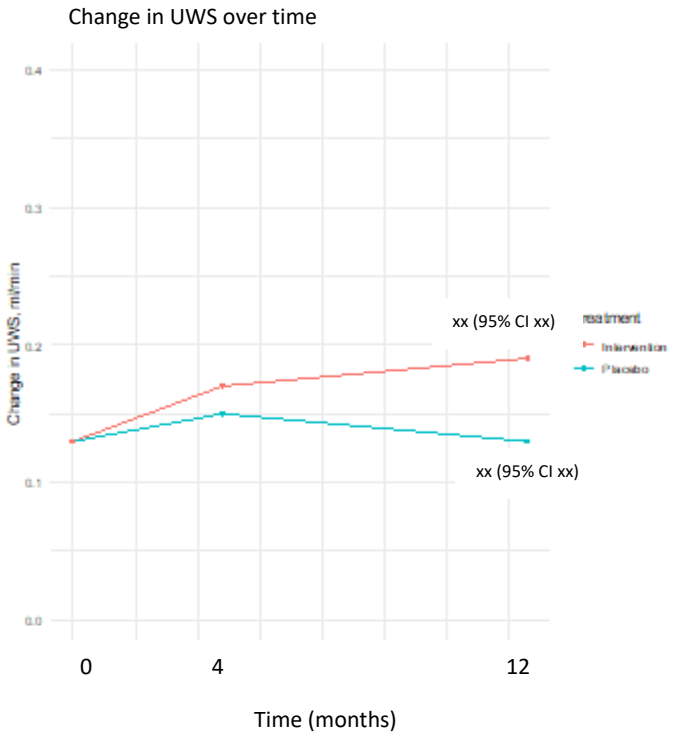
Figure 1. CONSORT Flow diagram illustrating the trial flow Patient Flow Throughout the 12 months follow-up in the MESRIX-III Randomized Clinical Trial



Abbreviation: ENT: Department of otorhinolaryngology, head and neck surgery

Figure 2. Simulated data: Change in salivary gland function measured over the 12 months period in **A**. Unstimulated whole saliva flow rate (UWS) **B**. Stimulated whole saliva flow rate (SWS).

A.



B.

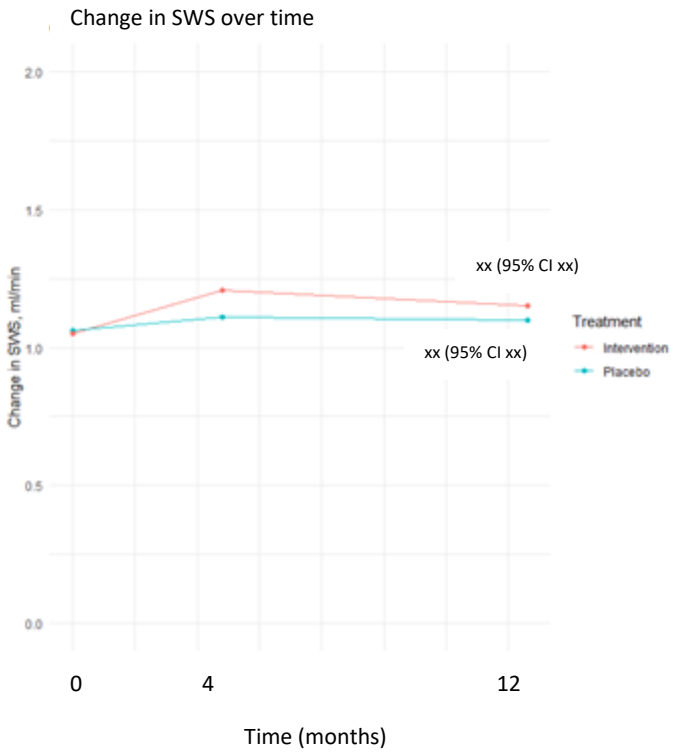


Figure 3. Simulated data: Change in salivary gland function measured over the 12 months period in the patient reported outcomes. **A.** XQ questionnaire. **B.** HNDR: domains for dry mouth evaluated by EORTC QLQ-H&N35; **C.** HNSS: domains for sticky saliva evaluated by EORTC QLQ-H&N35. HNSW: domains for swallowing evaluated by EORTC QLQ-H&N35

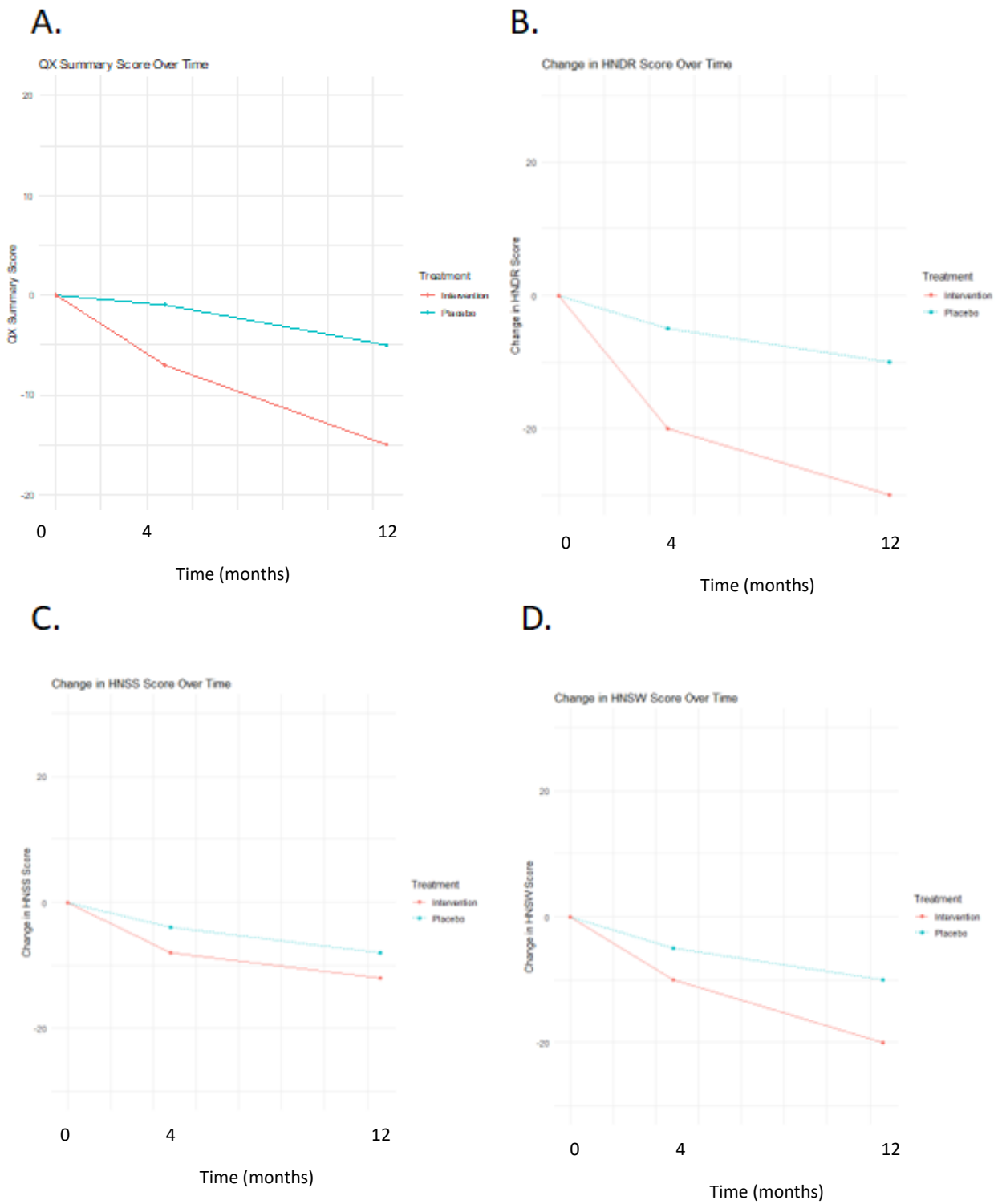


Table 2. Prespecified systematic harms; Safety profile up to 12 months from baseline.

Adverse events	**ASC group (n=60)**	**Placebo group (n=60)**	**Risk Difference (95%CI)**
Deaths, no (%)			
Serious adverse events, no (%)			
De novo donor specific antibodies, no. (%) Patients with persistent response from 4 to 12 m, no. (%)* Patients with increased response from 4 to 12 m, no. (%)* Patients with reduced response from 4 to 12 m, no. (%)* Patients with resolved response from 4 to 12 m, no. (%)*		NA	NA

*An increase or decrease is defined as a change in MFI sum of DSA $\geq$ 3000.

Table 3. Comparison of change from in secondary outcomes from baseline to 12 months follow-up (Trial secondary end point)

	ASC (n = 60)	**Placebo** (n = 60)	**Difference (95% CI)**	***P*-value**
Change From Baseline to 12 months				
Unstimulated saliva flow rate, ml/min				
Stimulated saliva flow rate, ml/min				
XQ-summary score (0-100)				
EORCT QLQ-H&N35 (score 0-100):				
HNDR				
HNSS				
HNSW				

Values will be least squares means with standard errors for each group; the difference between these will be reported with 95% confidence intervals (95%CIs). Estimates will be derived from the repeated measures, mixed effects models.

Supplementary Table 1. Immune response and development of HLA antibodies from baseline to 12 months in the ASC group.

Donor 1: HLA-A3,-; B18,37; Cw6,7; DR4,14; DR52, DR53; DQ5(1),8(3); DPB1*02:01,16:01

Donor 2: HLA-A2,11; B57,B62(15); Cw9(3),6; DR7,13(6); DR52,DR53N; DQ6(1),9(3); DPB1*04:01,-

Donor 3: HLA-A2,3;B7,-;Cw7,-;DR15(2),-;DR51;DQ6(1),-; DPB1*02:01,04:01

Pt ID no	Donor / Placebo	Donor-specific HLA antibodies (MFI)				Notes 4 months follow-up	Notes – 12 months follow-up
		HLA Antigen	Baseline MFI	4 months MFI	12 months MFI		
002	1	A3 B18 B37 DQ5	(-) 4000 2000 4000	3000 10000 5000 4000		De novo antibodies and preformed antibodies	
003	1	-	(-)	(-)			
007	1	-	(-)	(-)			
008	1	B37	2000	1000		Preformed antibodies	
010	1	-	(-)	(-)			
015	2	B57 B62	(-) (-)	2000 2000		De novo antibodies	
016	2	-	(-)	(-)			
018	2	A11	(-)	(-)			
020	1	-	(-)	(-)			
021	2	A2 B57 B62(15)	(-) (-) (-)	2000 6000 1000		De novo antibodies	
022	2	-	ND	(-)			
024	2	A2 B57 B62(15) Cw9(3)	(-) (-) (-) (-)	7000 14.000 2000 1000		De novo antibodies	
026	2	B57 B62(15)	(-)	2000 1000		De novo antibodies	
028	1	-	(-)	(-)			
029	1	-	(-)	(-)			
033	1	-	(-)	(-)			
034	1	-	(-)	(-)			
035	1	-	(-)	(-)			
037	3	A2 A3 B7	(-) (-) (-)	16000 7000 15000		De novo antibodies	-
038	3	-	(-)	(-)			
040	3	B7	(-)	1000		De novo antibodies	
041	3	-	(-)	(-)			
043	3	A3 DQ6	(-) 3000	2000 3000		De novo antibodies	
047	3	(-)	(-)	(-)			
049	3	A3 B7 DR15 DR51 DQ6(1)	(-) 3000 10000 11000 6000	10000 22000 10000 11000 6000		De novo antibodies and preformed antibodies	
053	3	-	(-)	(-)			
054	3	-	(-)	(-)			
059	3	A2	(-)	<2000		De novo antibodies	
061	3	A3 B7 DQ6(1)	(-) (-) 7000	12000 8000 7000		De novo antibodies	
062	2	-	(-)	(-)			

063	3	-	(-)	(-)			
066	3	-	(-)	(-)			
068	2	B62(15) Cw9	2000 2000	12000 9000		Preformed antibodies	
071	3	-	(-)	(-)			
073	3	-	(-)	(-)			
074	3	-	(-)	(-)			
075	2	A2 B57 B62(15) DQ6(1)	(-) (-) (-) 1000	2000 5000 1000 1000		De novo antibodies	
076	2		(-)	(-)			
080	3	-	(-)	(-)			
082	2	A2 A11 B57 B62(15)	(-) (-) (-) (-)	7000 2000 14000 5000		De novo antibodies	
083	3	-	(-)	(-)			
086	3	A3 B7	(-) (-)	1000 2000		De novo antibodies	
088	2	-	(-)	(-)			
089	3	A2	(-)	2000		De novo antibodies	
091	1	-	(-)	(-)			
092	3	A2 A3	(-) (-)	8000 6000		De novo antibodies	
095	3	-	(-)	(-)			
096	1	-	(-)	(-)			
100	1	-	(-)	(-)			
101	3	-	(-)	(-)			
105	3	-	(-)	(-)			
106	3	-	(-)	(-)			
107	3	A2 A3 B7	(-) (-) (-)	6000 2000 4000		De novo antibodies	
111	1	-	(-)	(-)			
112	1	-	(-)	(-)			
113	2	A2 B57	(-) (-)	2000 2000		De novo antibodies	
117	3	-	(-)	(-)			
120	1	Cw7	1000	(-)		Preformed antibodies	
122	3	A2 A3 B7	(-) (-) (-)	5000 7000 5000		De novo antibodies	
123	1	-	(-)	(-)			

*Missing data.

Abbreviations: MFI: normalized mean fluorescence intensity; MFI is approximate values (rounded to the nearest thousand); (-): negative (MFI < 1000 for Labscren Single Antigen, One Lambda, NBG<3.0 for HLA class I and NBG<4.0 for HLA class II for Labscreen Mixed, One Lambda)

Supplementary Table 2. Sensitivity analysis, missing data replaced using a simplistic non-responder imputation. Comparison of change from in secondary outcomes from baseline to 12 months follow-up (Trial secondary end point)

	ASC (n = 60)	**Placebo** (n = 60)	**Difference (95% CI)**	***P*-value**
Change From Baseline to 12 months				
Unstimulated saliva flow rate, ml/min				
Stimulated saliva flow rate, ml/min				
XQ-summary score (0-100)				
EORCT QLQ-H&N35 (score 0-100):				
HNDR				
HNSS				
HNSW				

Values will be least squares means with standard errors for each group; the difference between these will be reported with 95% confidence intervals (95%Cis). Estimates will be derived from the repeated measures, mixed effects models.

References

1. Lynggaard CD, Grønhøj C, Jensen SB, et al. Long-term Safety of Treatment with Autologous Mesenchymal Stem Cells in Patients with Radiation-Induced Xerostomia: Primary Results of the MESRIX Phase I/II Randomized Trial. *Clin Cancer Res*. 2022;28(13):2890-2897. doi:10.1158/1078-0432.CCR-21-4520

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Supplementary 6. Study Representativeness Table.

Cancer type(s)/subtype(s)/stage(s)/condition	Head and Neck Cancer
Considerations related to:	
Sex	In Demark, overall, 65% of patients with head and neck cancer are men, and up to 75% in specific subtypes such as oropharyngeal cancer (OPSCC), but the incidence in women is rising. Men have a poorer survival than women with a relative 5-year overall survival of 67 % (95% CI 64-70%) compared to 62 % (95% CI 59-65%) in female in the period from 2017-2021.
Age	Most patients (60%) with head and neck cancer are over 60 years old at diagnosis and a mean age of 62.6 years (95% CI 62.3 to 62.8) Patients with head and neck cancer driven by human papillomavirus (HPV) are usually younger, however an increasing incidence among elderly have been observed.
Race/ethnicity	The incidence of head and neck cancer differ by ethnicity, which is also associated with overall survival. It has been shown that black race is associated with a worse survival, but the mechanisms are poorly understood. Differences in socioeconomic status, clinical and treatment-related factors might contribute to the observed differences.
Geography	Globally, more than 900,000 get head and cancer each year and with an increasing incidence. The rise in head and neck cancer is primarily driven by HPV-associated oropharyngeal cancer. The HPV prevalence vary greatly among diverse geographical areas, and the highest prevalences are observed in Western countries. In Denmark, approximately 65% of oropharyngeal cancers are HPV-positive.
Other considerations	Head and neck cancer is a heterogenous group of cancers, but is most often squamous cell carcinomas arising from the mucosal

	epithelium. Historically, head and neck cancers are associated with alcohol and tobacco consumption, but for the last decades infection with HPV has been associated with oropharyngeal cancer, especially in the palatine tonsils and base of the tongue. Most head and neck cancers are treated with radiotherapy with or without concomitant chemotherapy and/or surgery, most often multimodal. The overall survival is best for HPV-positive oropharyngeal cancer, with a 5-year overall survival of 81 % (95% CI 80-83%) compared to 62% (95% CI 59-65%) for all head and neck cancers.
Overall representativeness of this study	The median age in this study population was 61 years, which corresponds to the mean age in the general head and neck cancer population. Also, most patients in this study population were males (73%) which align with the sex distribution in general ranging from 65-75% depending on anatomical subsite and HPV-status. Most patients had oropharyngeal cancer (87%), with the majority being HPV-positive. With higher survival rates, these patients have more time to experience treatment-related side effects, potentially leading to a prolonged burden of these adverse effects throughout their lives.

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Title

No Changes in the Salivary Proteome Composition Detected After Mesenchymal Stem Cell Therapy for Radiation-Induced Hyposalivation in Head and Neck Cancer Patients: A Randomized, Phase 2 Trial

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Conflict of interest

Lynggaard, Grønhøj, and von Buchwald are co-inventors on patent application for “Stem cell therapy for patients with salivary gland dysfunction”, PCT/EP2020/053878, owned by Rigshospitalet, Copenhagen University Hospital and University of Copenhagen.

Kastrup, Ekblond, and Haack-Sørensen hold a patent application for the investigated stem cell product “Stem cell therapy based on adipose-derived stem cells”, WO2017068140. None of the other co-authors declare conflicts of interest.

Keywords

Mesenchymal stem/stromal cells, mesenchymal stromal cells, MSC, xerostomia, hyposalivation, radiotherapy, proteomics, proteome.

Author contributions

ALFC: Conceptualization, data curation, funding acquisition, formal analysis, investigation, methodology, writing–original draft, project administration. **KKJ:** Conceptualization, data curation, funding acquisition, investigation, methodology, writing–review and editing, project administration **RJC:** Conceptualization, formal analysis, methodology, writing–original draft. **JK:** conceptualization, writing–review and editing. **DB:** Conceptualization, writing–review and editing**. CDL:** Conceptualization, methodology, writing–review and editing**. CG:** Conceptualization, methodology, supervision, investigation, writing–review and editing**. CVB:** Conceptualization, methodology, supervision, investigation, writing–review and editing**.**

Abstract

Background

Observations indicate that the salivary proteome may change following intraglandular adipose-derived mesenchymal stem cell (ASC) therapy. The aim of this study was to compare the salivary proteome four months following ASC therapy to placebo in previously irradiated head and neck cancer patients from a randomized trial.

Methods

120 patients were randomized 1:1 to receive either ASC therapy or placebo (Crystor10). Unstimulated whole saliva samples were collected at baseline and four months and analyzed with mass spectrometry-based proteomics. The primary endpoint was change in the salivary proteome following ASC therapy compared to placebo at four months. Results were visualized with principal component analysis and volcano plots, and significance was evaluated with a double-sided t-test.

Results

In total, 178 salivary samples from 89 previously irradiated head and neck cancer patients were collected with a total of 1510 proteins identified. In the ASC group, 1438 different proteins (mean 890 per sample; range: 532- 1099) were identified compared to 1432 different proteins in the placebo group (mean of 900 per sample; range: 653-1067). No significantly differentially expressed proteins were identified between the groups at four months. Several proteins upregulated in healthy saliva were insignificantly enriched in the ASC group compared to placebo.

Conclusions

We detected no significant differences in the salivary proteome four months following intraglandular ASC therapy compared to placebo in irradiated patients. While several proteins associated with healthy saliva were non-significantly enriched in the ASC therapy group, their levels remained below those observed in healthy individuals, suggesting a partial effect in maintaining salivary health.

Plain language summary

Dry mouth syndrome and reduced saliva production are the most common side effects following radiotherapy in the head and neck area, and no successful treatment options are available. Injections with stem cells in the salivary glands have shown a potential to restore saliva production and induce changes in the salivary protein composition related to regeneration. We compared the salivary protein composition following stem cell treatment to placebo in irradiated head and neck cancer patients. The results showed no significant change in the protein composition after stem cell treatment, though there was a slight increase in healthy salivary proteins, suggesting partial restoration.

Introduction

Worldwide 900,000 patients are diagnosed with head and neck cancer every year, and most patients undergo radiation-based therapy[1,2]. Despite, that intensity-modulated radiation therapy aims to reduce toxicity to healthy tissue, the salivary glands are often damaged following radiation therapy (RT)[3–5]. RT induces complex damages to the salivary glands encompassing inflammation, loss of salivary-producing acinar and progenitor cells, and fibrosis[6,7]. This may ultimately lead to salivary gland hypofunction and reduced salivary production, or dry mouth syndrome, xerostomia[8,9]. Changes in the saliva composition are further observed with alterations in the salivary proteins and protein abundance[9–12]. Consequently, RT-induced salivary gland hypofunction reduces overall oral health with increased risk of oral infections, impairs speech and swallowing, and impacts the overall quality of life in previous head and neck cancer patients[13,14]. There exist no available disease-modifying treatment strategies for hyposalivation, emphasizing the need for more treatment possibilities[15–17].

Mesenchymal stem cells (MSCs) possess various supportive and regenerative functions rendering them a potential therapeutic agent for the repair of salivary gland damage caused by RT[18,19]. Since MSCs can easily be isolated from various tissues including adipose tissue they offer a simply available, off-the-shelf, cell-based therapy for clinical use[20,21].

We have demonstrated that intraglandular adipose-derived mesenchymal stromal cells (ASCs) have the potential to enhance the salivary flow rate and improve the quality-of-life (QoL) in patients with previous head and neck cancer suffering from radiation-induced hyposalivation[22–24], although a superior effect of ACSs compared to placebo was not established in our latest study[22].

The mode of action of intraglandular ASC therapy remains unclear, but in our previous MESRIX-II study, we found that intraglandular ASC therapy modified the stimulated salivary proteome, leading to an increase in proteins linked to tissue regeneration, although not restored to normal after four months[25]. However, this has not been validated in a larger clinical trial.

Therefore, the aim of this study was to evaluate the change in the salivary proteome in unstimulated whole saliva (UWS) four months after intraglandular allogeneic ASC therapy compared to placebo in previous head and neck cancer patients with radiation-induced salivary gland hypofunction in a randomized, double-blinded, placebo-controlled trial. Secondary aims were to 1) evaluate the changes in the salivary proteome in the group receiving ASCs from baseline to four months 2) evaluate the change in the salivary proteome composition in ASC-treated subgroups associated with improved clinical effect of ASC therapy and 3) characterize the salivary proteome following RT at baseline in both groups compared to healthy controls. Our study did not demonstrate a change in the composition of salivary proteome following intraglandular ASC therapy compared to placebo in irradiated head and neck cancer patients. Although insignificant, we did observe a slight enrichment in important salivary proteins in the ASC group which are also upregulated in the healthy salivary proteome, indicating a partial saliva restoration.

Method and materials

Details on the study design, setting, population, sample size, randomization, blinding, and intervention were previously described in detail[22,26]. Additionally, stimulated saliva samples from ten healthy controls were obtained from our previous study[22]. Analysis of the salivary proteome was recorded as a pre-specified secondary endpoint, and a study protocol was published[27].

Study design

The study was a sub-study to the randomized, placebo-controlled, double-blinded trial investigating the effect on salivary gland function intraglandular ASCs treatment for radiation-induced hyposalivation in previous head and neck cancer patients[22,26]. The study was approved by the Danish Data Protection Agency (protocol number P-2020-1164), the National Ethics Committee protocol number: (Protocol number: 1802872), and the Danish Medical Agency (EudraCT: 2018-000348-24) and registered at the ClinicalTrials.gov database (NCT04776538)[22,26].

The study was conducted in coherence with the protocol and complied with the Declaration of Helsinki and monitored by the Good Clinical Practice (GCP) unit at the University of Copenhagen. Written informed consent was obtained from all study participants prior to enrolment. The study followed the Consolidated Standards of Reporting Trials (CONSORT) reporting guidelines for randomized clinical trials[28].

Study setting and participants

The study was conducted at the Department of Otorhinolaryngology, Head and Neck Surgery and Audiology, Rigshospitalet, Denmark, as previously described in detail[22,26].

Eligible patients were previously irradiated head and neck cancer patients with clinically observed hyposalivation of UWS 0.05-0.25 ml/min and with two years without recurrence, between 18-75 years, without xerogenic medicine, and informed consent. Patients were excluded if they had other cancers in the last four years, other salivary gland disease, were pregnant or breastfeeding, were smokers, or had an alcohol abuse[22,26].

Sample size, randomization, and blinding

Based on our previous study, 120 patients were included[22,26]. The sample size was developed for the primary endpoint in the main study, which was unstimulated salivary flow rate. Allocation of intervention and blinding were previously described in detail[22,26]. The follow-up at 4 months was kept blinded to participants and all study personnel involved in both data collection and data analysis.

Intervention

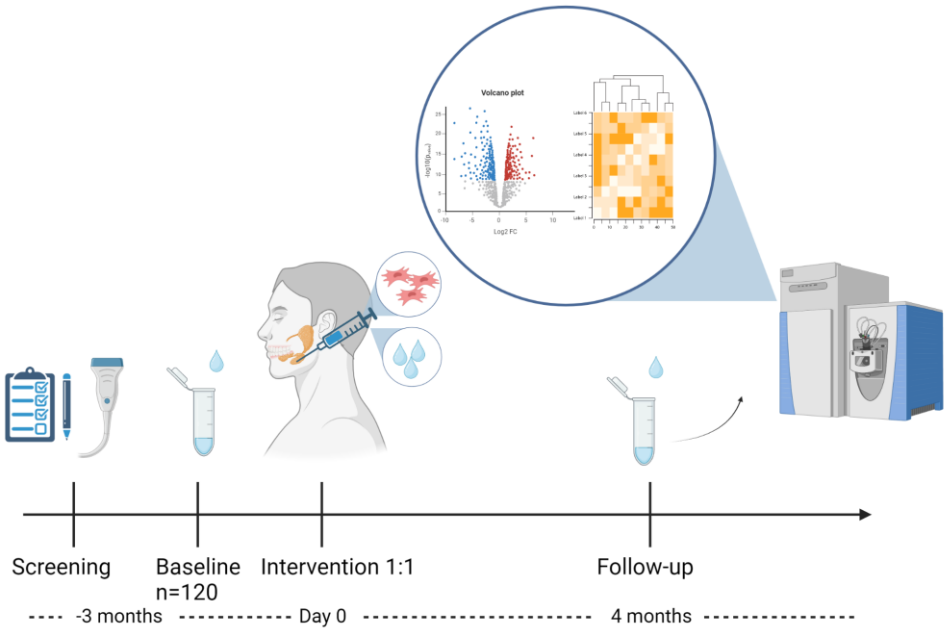
Patients received intraglandular ASC therapy consisting of 0.5 mL of ASCs ($25x10^6$ ASCs per gland) or placebo, which was CryoStor10 (BiolifeSolutions) containing 10% dimethyl sulfoxide (DMSO). The injections were performed ultrasound-guided by free hand into the submandibular glands. Details on the interventions are previously described in detail[22,26].

Saliva collection

UWS was collected by sialometry using the spitting method over 10 minutes at baseline and at four months after intervention. The sialometry was performed at the same time of the day at subsequent collections throughout the study. UWS was collected for 10 min. The saliva samples were immediately stored on dry

ice and afterward stored in Cryotubes at -80 C until analysis. Further details on the sialometry are previously described in detail[22,26]. See study flow in **Figure 1**.

Figure 1. **Study flow.** A total of 120 patients were included and randomized 1:1 to receive either intraglandular ASC therapy or placebo. UWS samples were collected at baseline and four months after intervention and subsequently underwent proteomic analysis using LC-MS/MS.



**Created with Biorender.*
Abbreviations: ASC, adipose-derived mesenchymal stem cell; LC-MS/MS, nanoscale liquid chromatography-tandem mass spectrometry; UWS, unstimulated whole saliva.

Sample preparation
Sample preparation was modified from https://doi.org/10.1007/978-1-4939-3049-4_17 and is previously described[27]. Briefly, 500 µL UWS was lysed with 500 µL 95 °C warm lysis buffer (8 M Guanidinium hydrochloride (GuHCl), 5 mM tris(2-carboxyethyl) phosphine (TCEP), 10 mM chloro-acetamide (CAA), 100 mM Tris–HCl pH 8.5) and heated for 10 min. at 95 °C followed by sonication. Subsequently, 200 µg protein from each sample was diluted with 25mM Tris-HCL (pH 8.5, 5x) and digested with Tryspin (MS grade; Thermo) overnight at 37 °C in a 1:50 w/w ratio. To stop digestion, 10% trifluoroacetic acid (TFA) was added to a final concentration of 1%. Sep-Pak C18 (Waters) with activation by 100% acetonitrile (ACN) and equilibration with 0.1% TFA was used to desalt and concentrate peptides. Samples were then washed with 0.1% TFA and eluted with 40% ACN followed by 60% ACN. Lastly, peptides were dried in a SpeedVac for 1 h at 60 °C and resuspended in 50 µL 5% ACN in 0.1% TFA.

Mass spectromectry analysis

The mass spectrometry analysis is previously described[27]. In short, approximately 1 ug peptide solution was analyzed by online nanoscale liquid chromatography-tandem mass spectrometry (LC-MS/MS). Peptides were separated on a 50 cm C18-column (Thermo EasySpray ES804A) using an Ultimate 3000 system (Thermo Scientific) at 40 °C. A gradient flow rate of 250 nl/min was used, transitioning from 1% to 40% MS buffer B over 80 minutes, followed by a 10-minute step to 95%, a 5-minute hold, and then re-equilibration at 5% for 10 minutes. MS buffer A consisting of 0.1% FA and MS buffer B of 90% ACN, 0.1% FA. The Q Exactive Plus instrument (Thermo Scientific) operated in data-dependent acquisition mode with a top 20 Higher-energy Collisional Dissociation (HCD)-MS/MS method (scan range 375–1500 m/z, full-scan resolution 70,000 m/z, AGC target of 3e6, maximum injection time (IT) of 15 ms). Peptides were fragmented with a normalized collision energy of 30, dynamic exclusion of 10 s, excluding unassigned ions, and those with a charge state of 1, 6–8. MS/MS resolution was set at 17,500 m/z, with an AGC target of 1e5 and a maximum IT of 45 ms.

Data analysis

Data analysis is previously described[27]. The raw LC-MS/MS data files were processed with MaxQuant version 2.1.4.0 using default settings and label-free quantification. In brief, variable modifications included oxidation (M), protein N-termini acetylation, and met-loss, with cysteine carbamidomethyl as a static modification. A 1% false discovery rate and match between runs were applied. MaxQuant output was analyzed with Perseus version 1.6.5.0, excluding proteins with fewer than two peptides for quality assurance. Data was searched against reviewed human database from Uniprot downloaded May 2019, containing 73911 entries, uniport.org, https://doi.org/10.1093/nar/gkac1052. PCA plots were done with proteins identified in 50% of all the relevant samples, and with imputation from normal distribution. Volcano plots were created with proteins identified in 50% of all the relevant samples, and double-sided t-test. Salivary proteins that we previously identified to be upregulated in healthy saliva (Cystatin-D, -D, -SA, -SN, Glutaredoxin-1, Histatin-1, LIpocalin-1, Statherin) was identified as an indicator of repairment[25]. Enriched biological terms were analyzed in the Database of Annotation, Visualization and Integrated Discovery (DAVID) 2021 version v2023q4. Functional annotation included Gene Ontology Biological Process (GO:BP) using cluster analysis. Enriched clusters were analyzed in a descriptive manner and compared to salivary proteomic profiles from healthy controls obtained in our previous study[25].

A non-responder analysis was performed to compare included and excluded patients in key baseline characteristics (intervention, age, gender, smoking status, head and neck cancer anatomical location, early stage I-II, mean radiation dose to the four large salivary glands, and duration since radiotherapy). Categorical variables were evaluated for significance with Person's Chi Square test, while continuous data were evaluated using *t-test*. The statistics were performed in R statistics version 4.1.3.

Results

Study characteristics and clinical outcome

The results of the clinical characteristics and clinical outcome are previously described[22]. Briefly, no differences were observed between the group receiving ASC therapy and placebo in baseline characteristics[22]. Median age was 61 years (range: 43-75 years) with the majority being male (73%) and most patients had an HPV-positive/p16-positive oropharyngeal cancer (78%). Most patients were diagnosed at early stages (I/II: 74%) and received multimodality treatment (chemoradiotherapy: 85%) with a mean duration since RT of 5.7 years.

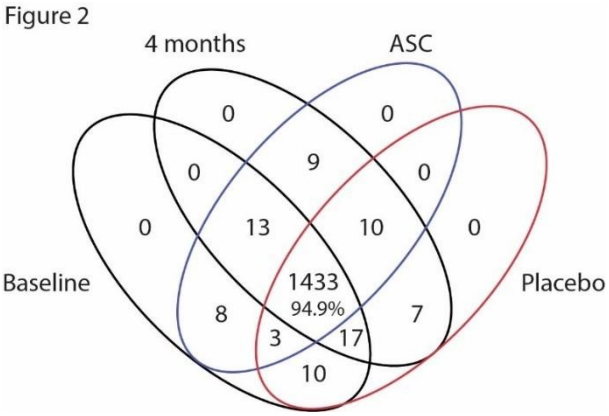
As previously published, the study revealed no significant clinical effect on the UWS in the ASC group compared to placebo, but an increase within the ASC group was observed[22]. Sub-analyses revealed that smoking, higher mean radiation dose to the four major salivary glands, and development of donor-specific antibodies (DSAs) were associated with less improvement in UWS.

Protein expression

In total, 238 samples of UWS from 119 previously RT head and neck cancer patients (one per patient at baseline and four-month follow-up) were collected. One patient had an UWS of 0 mL/min at the four-month follow-up visit and was therefore excluded from the analysis. Two outliers were removed from the dataset. Due to inconsistent sample preparation, additionally 28 patients were excluded from the dataset. This resulted in a dataset consisting of 89 patients. A non-responder analysis revealed that excluded patients were significantly more smokers than included patients but were comparable on all other baseline characteristics, data not shown.

Across all samples (n=178), 1510 proteins were identified. From the baseline samples, 1484 different proteins were identified with a mean number of 889 per sample (range: 523-1165). At four months after the intervention, 1438 different proteins were identified with a mean number of 890 per sample (range: 532- 1099) in the ASC group, compared to 1432 different proteins were identified with a mean number of 900 per sample (range: 653-1067) in the placebo group. See **Figure 2**.

Figure 2. **Venn diagram of identified proteins in both the ASC group and in the placebo group at four months**. In total, 1510 proteins were identified across all samples. Most proteins were shared between the groups (1433, 95%).

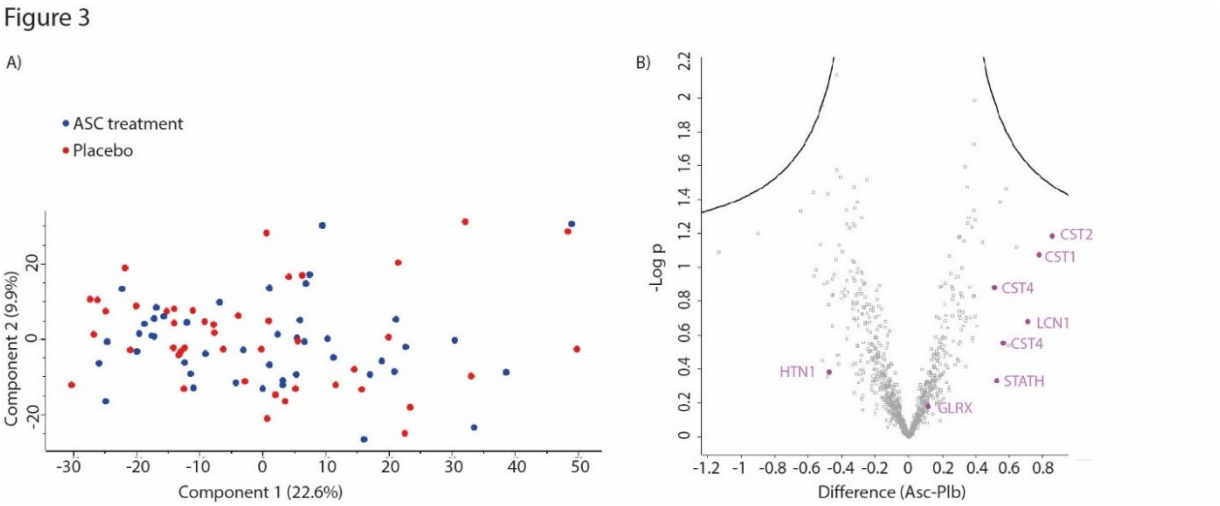


Abbreviations: ASC, adipose-derived mesenchymal stem cell.

The salivary proteome following ASC therapy compared to placebo

The PCA shows no clear separation of samples from patients receiving ASC therapy compared to patients receiving placebo at four months following the intervention. See **Figure 3A**. The Volcano plot in **Figure 3B** illustrates the distribution of the proteins in the ASC group compared to the placebo group and shows no significantly differentially expressed proteins in the two groups. However, proteins that we previously identified to be upregulated in saliva samples from healthy controls[25], trended to be enriched in the ASC group compared to the placebo group, see **Figure 3B**. These proteins included Cystatin-S, Cystatin-D, Cystatin-SA, Cystatin-SN, Glutaredoxin-1, and Lipocalin-1, while Histatin-1 was downregulated in the ASC group. See **Table 2**.

Figure 3. **Comparison of patients receiving ASC therapy compared to placebo at four months**. **A.** PCA demonstrating no clear separation. **B.** Volcano plot illustrating no differentially expressed proteins. Proteins that trended to be significantly differentially expressed between the ASC group and the placebo group are highlighted.



Purple circle = marked proteins associated with healthy saliva.
Abbreviations: ASC, adipose-derived mesenchymal stem cell; PCA, principal component analysis.

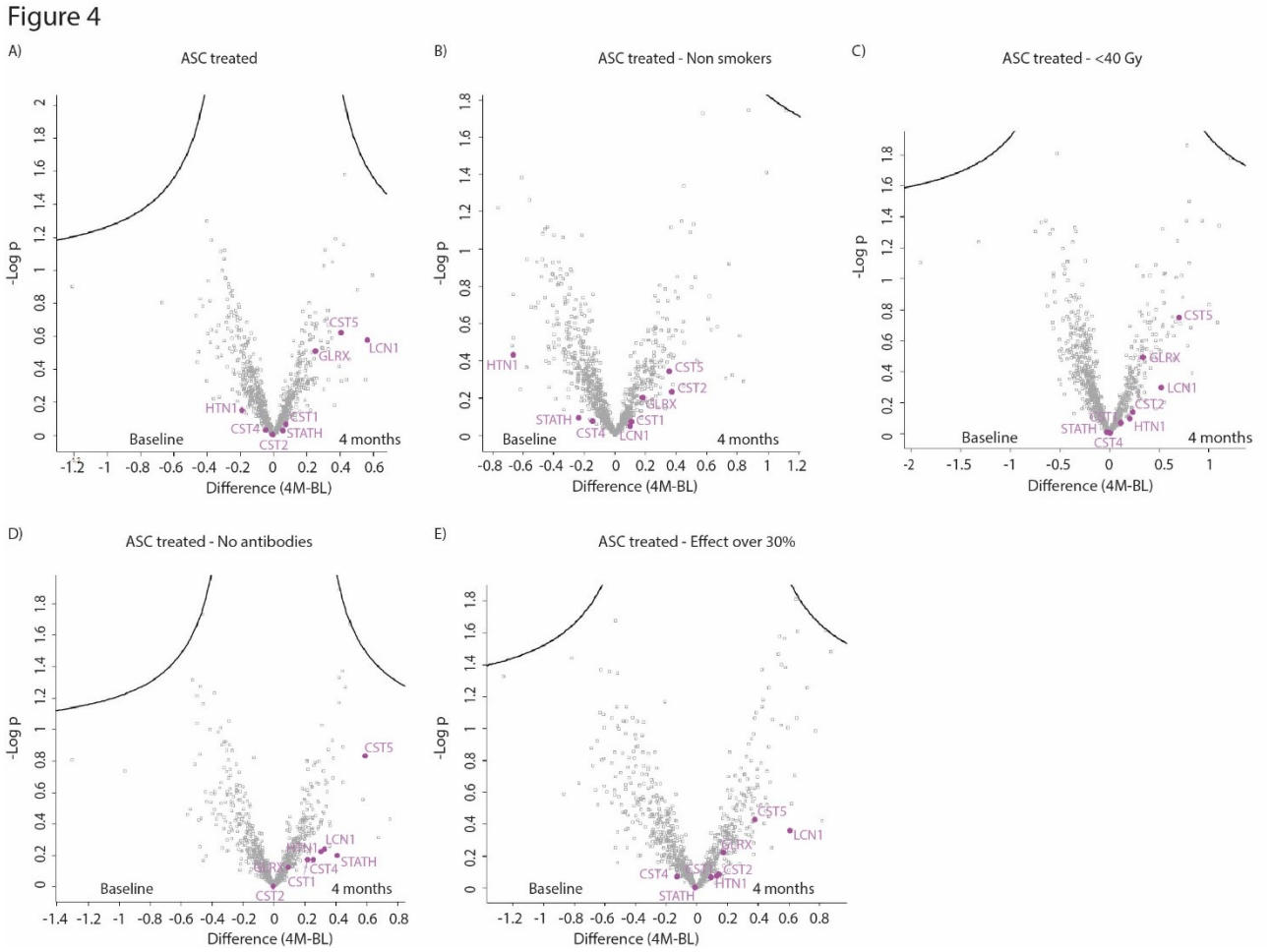
Table 2. **Proteins upregulated in healthy saliva and at four months in patients receiving ASC therapy trending to be differentially expressed compared to placebo**.

Accession	Gene name	Proteins with higher or lower intensity in the ASC group vs placebo	-LOG (P value)	Difference
P28325	CST5	Cystatin-D	0.883	0.512
P01036	CST4	Cystatin-S	0.555	0.566
P09228	CST2	Cystatin-SA	1.187	0.857
P01037	CST1	Cystatin-SN	1.077	0.778
P35754	GLRX	Glutaredoxin-1	0.181	0.117
P15515	HTN1	Histatin-1	0.382	-0.475
P31025	LCN1	Lipocalin-1	0.680	0.710
P02808	STATH	Statherin	0.332	0.526

Sub-analysis of the salivary proteome following ASC therapy

Within the ASC group, no differentially expressed proteins were observed following ASC treatment at four months compared to baseline. No differences in the salivary proteins upregulated in healthy saliva were observed either. See **Figure 4A**. While a less increase in the clinical outcome UWS was associated with smoking, higher mean radiation dose, and development of DSAs in the main study [22], no differences in the salivary proteome were observed following ASC treatment at four months compared to baseline for never smokers, patients receiving a mean radiation dose the large salivary glands < 40 Gy, or patients who did not develop DSAs, see **Figure 4B-D**. However, proteins that we previously identified to be upregulated in healthy controls[25], tended to be enriched in the group who did not develop DSAs at four months compared to the baseline, See **Figure 4D**. These proteins included Cystatin-D, Cystatin-SN, Cystatin-SA, Glutaredoxin-1, Histatin-1 and Lipocalin-1. A difference in the salivary proteome was not observed within the group who experienced a clinical effect on UWS of 30% or more either, see **Figure 4E**.

Figure 4. **Comparison of patients receiving ASC therapy at baseline and four months after intervention. A.** Volcano plot illustrating no differentially expressed proteins in patients receiving ASC therapy. **B.** Volcano plot illustrating no differentially expressed proteins in patients with no history of smoking and who received ASC therapy. **C.** Volcano plot illustrating no differentially expressed proteins in patients with a mean radiation dose to the four large salivary glands <40 Gy and who received ASC therapy. **D.** Volcano plot illustrating no differentially expressed proteins in patients who did not develop DSAs and who received ASC therapy. **E**. Volcano plot illustrating no differentially expressed proteins in patients with an effect on clinical evaluation of the unstimulated flow rate of 30% or more and who received ASC therapy.



Purples circle = marked proteins associated with healthy saliva.
Abbreviations: ASC, adipose-derived mesenchymal stem cell; BL, baseline; DSAs, donor-specific antibodies; Gy, Gray; M, months.

The salivary proteome in irradiated head and neck cancer patients compared to healthy controls
Descriptive functional annotation analysis of GO:BP revealed that the most enriched clusters in irradiated patients compared to healthy controls were metabolic processes (score 18.9 versus 9.2), immune response

(score 19.7 versus 22.7), oxidative stress and detoxification processes (score 12.5 versus 9.4), injury response (score 8.2 versus 7.3), and antimicrobial response (score 5.5 versus 12.1). See **Table 3**.

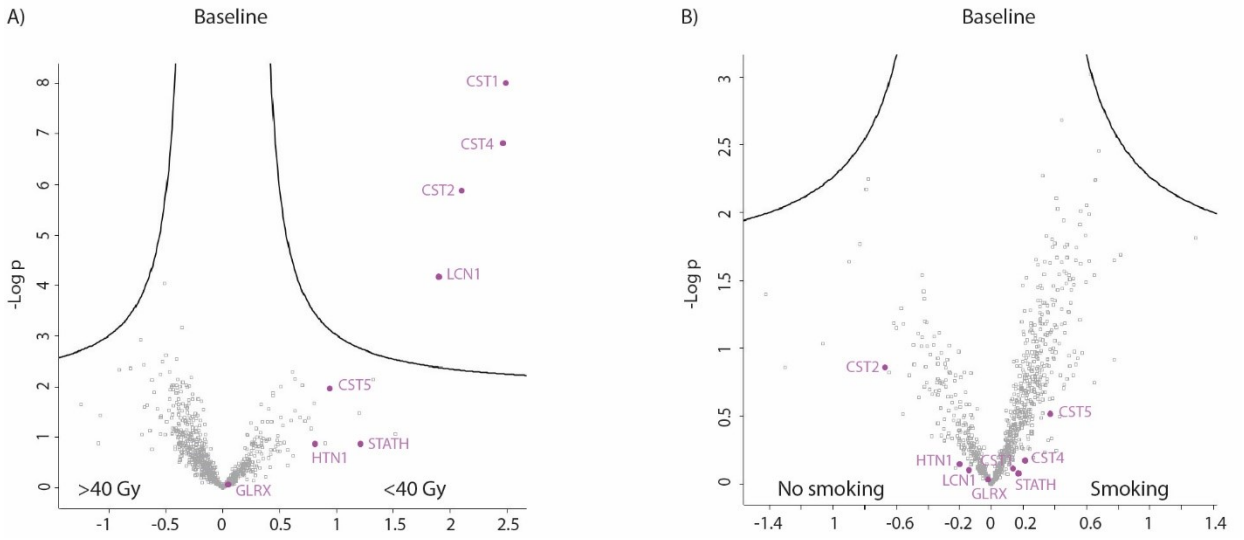
Table 3. List of enriched proteins identified in saliva from irradiated patients and healthy controls.

Gene ontology	**Baseline, (n=89)**		**Healthy controls, (n=10)**	
Biological function (level 3)	**Enrichment score, count**	***P* value**	**Enrichment score, count**	***P* value**
Metabolic processes	**18.9**		**9.2**	
Phosphorus metabolic process	206	5.7E-9	143	3.2E-2
nucleobase-containing small molecule metabolic process	139	1.2E-31	95	4.2E-14
purine-containing compound metabolic process	123	4.7E-31	84	3.0E-14
carbohydrate derivate biosynthetic process	72	3.9E-4	46	3.1E-1
Immune response	**19.7**		**22.7**	
Defense response	226	5.4E-23	212	2.5E-25
Innate immune response	129	3.1E-15	123	1.7E-17
response to bacterium	113	2.5E-12	115	3.9E-23
humoral immune response	-		72	2.9E-17
Oxidative stress and detoxification processes	**12.5**		**9.4**	
cellular oxidant detoxification	36	1.1E-16	31	7.9E-14
reactive oxygen species metabolic process	36	4.8E-13	28	6.5E-9
Injury response	**8.2**		**7.3**	
response to wounding	79	6.0E-12	71	3.0E-11
blood coagulation	39	61.3E-8	34	1.8E-7
regulation of body fluids	60	3.6E-8	49	6.2E-6
Antimicrobial response	**5.5**		**12.1**	
response to fungus	19	1.2E-6	21	4.7E-9
killing of cells of another organism	21	5.5E-6	31	1.4E-14

For irradiated patients at baseline, significantly differentially expressed proteins were observed in patients who received a lower mean radiation dose to the four salivary glands (<40 Gy) as compared to those who received a higher dose. Cystatin-SN (difference 2.45, -log *p*-value 6.85), Cystatin-S (difference 2.37, -log *p*-value 5.54), Cystatin-SA (difference 1.91, -log *p*-value 4.36), and Lipocalin-1 (difference 1.77, -log *p*-value 3.56) were significantly upregulated in patients with a lower mean radiation dose. See **Figure 5A.** No differences were observed in patients with no history of smoking as compared to smokers. See **Figure 5B.**

Figure 5. Comparison of irradiated patients at baseline. A. Volcano plot illustrating significantly differentially expressed proteins in irradiated patients who received a mean radiation dose to the four large salivary glands < 40 Gy as compared to > 40 Gy. **B.** Volcano plot illustrating no differentially expressed proteins in irradiated patients with no history of smoking as compared to smokers.

Figure 5



Purple circle = significantly upregulated proteins.
Abbreviations: Gy, Gray.

Discussion

This is the largest randomized, placebo-controlled trial investigating the salivary proteome following intraglandular ASC therapy compared to placebo in previous head and neck cancer patients with radiation-induced hyposalivation. We did not find any significant differences in the salivary proteome between patients receiving ASC therapy compared to placebo four months following intervention.

Our results align with the primary endpoint of our study, which was clinical change in UWS, as the increase in UWS in the ASC group was 0.04 mL/min compared to 0.01 mL/min in the placebo group (difference of 0.03 mL/min, p=0.11)[22]. Yet, we previously found significant changes in the salivary proteome following ASC therapy in our pilot study, MESRIX-II, with upregulation of major salivary proteins as well as proteins involved in cell growth, immune system, and regeneration[25]. However the study was small (n=10) and not randomized, while this study included 120 irradiated head and neck cancers and was compared to placebo. We found that patients who received ASC therapy tended to have a salivary proteome more similar to that of healthy saliva compared to placebo, with insignificant upregulation of Cystatin-S, Cystatin-D, Cystatin-SA, Cystatin-SN, Glutaredoxin-1, and Lipocalin-1 which have shown to be significantly upregulated in healthy individuals[25]. Still, the levels did not reach those observed in healthy saliva as compared to irradiated head and neck cancer patients[10,25]. Both cystatins, statherins, and histatins are components of the first-line immune defense in the oral cavity, e.g., is cystatin-S among others associated with oral gingivitis and caries while histatins are important antifungal proteins[29–31]. Cystatin-D also impacts the subjective feeling of oral dryness, while statherins are important for calculus formation, and Lipocalin-1 is involved in taste reception[31–33].

Some patients receiving ASC therapy might have a more favorable clinical response to ASC therapy, as less improvement in the clinical outcome UWS was seen in patients with a history of smoking, who received a higher mean radiation dose, and who developed DSAs as shown in our previous study[22]. Therefore, we hypothesized that changes in the salivary proteome following ASC therapy would be more evident in patients with no history of smoking, who received a lower mean radiation dose (<40 Gy), who did not develop DSAs and with a substantial clinical effect (>30% increase in UWS). However, no differences in the salivary proteome within these subgroups were observed, but in ASC-treated patients who did not develop DSAs, a non-significant upregulation of Cystatin-D, Cystatin-SN, Cystatin-SA, Glutaredoxin-1, Histatin-1 and Lipocalin-1 was observed. This indicates that immunological tolerance without development of DSAs might be important in restoring the saliva composition.

In line with results from our MESRIX-II study[25], we found notable differences in the functional annotation of proteins identified in the UWS in irradiated patients compared to healthy individuals. An increased metabolic activity was identified in both groups, emphasizing the metabolic and biosynthetic processes in saliva necessary for e.g., enzyme synthesis. Yet, the metabolic processes were more increased in irradiated patients, which could reflect increased metabolic activity as cells attempt to repair and recover from radiation-induced damage. Also, enrichment in oxidative stress was found possibly due to radiation exposure, which is known to generate reactive oxygen species that lead to oxidative stress[34]. Both groups had an enriched immune and injury response, which is expected as saliva plays a crucial role in defending against pathogens[35]. Hynne et al., also identified differentially expressed salivary proteins involved in inflammation, host cell injury, oxidative stress response activation, and tissue restoration in irradiated patients compared to healthy controls[10]. A recent prospective study also identified increased levels of inflammatory protein markers following RT as compared to before RT[12]. Interestingly we found that patients who received a mean radiation dose to the four large salivary glands below 40 Gy had significantly increased levels of Cystatin-SN (more than twofold) Cystatin-S (more than twofold), Cystatin-SA and Liocalin-1. All four proteins are associated with a healthy salivary proteome[11,25], and are involved in maintenance of the overall oral health and taste reception[31,33]. This emphasizes how crucial it is to administer RT while minimizing dose and/or protecting the salivary glands when feasible to protect not only salivary flow rates but also salivary quality[5,36].

Several limitations of this study need to be addressed. Not all samples were included in the proteomic profiling due to inconsistent sample preparation, and significantly more of the excluded patients were smokers. Smoking was significantly associated with a decrease in UWS[22], and could potentially impact the salivary proteome as well. Second, we found substantial interpersonal differences already present at baseline, making it difficult to identify small changes induced by ASC therapy. Additionally, we compared the unstimulated salivary proteome in irradiated patients with the stimulated salivary proteome in healthy controls. Unstimulated and stimulated saliva differ in essential aspects[37,38]; which might contribute to the differences observed in this study. We did not evaluate or account for the oral status at baseline which also influences the salivary proteome[39,40]. Lastly, the study sample size was powered to detect a clinical change in salivary flow rate and not changes in the salivary proteome composition[26].

As the salivary proteins levels displayed great interpersonal heterogeneity already present at baseline, the future of intraglandular ASC therapy might be reserved for a smaller group of head and neck cancer patients, and future studies should be powered to investigate patient-specific factors. Early intervention

following RT might also enhance the effect of ASC therapy, since the salivary gland damage may be more reversible at this stage. Also, repeated ASC treatment could be beneficial as demonstrated in e.g., neurological disease[41,42].

In conclusion, we found no significant differences in the salivary proteome four months following intraglandular ASC therapy in previous head and neck cancer patients with radiation-induced hyposalivation compared to placebo. However, while several proteins upregulated in healthy saliva were non-significantly enriched in the ASC therapy group, their levels remained below those observed in healthy individuals. This suggests that ASC may have a partial effect on maintaining salivary health.

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