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CANCER THERAPY INDUCED MUCOSITIS

MOLECULAR CHARACTERISTICS INFLUENCING PRESENCE AND SEVERITY OF MUCOSITIS DURING CANCER THERAPY

BY
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DISSERTATION SUBMITTED 2017

AALBORG UNIVERSITY
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Dissertation submitted

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III. **Mette Marcussen, Mads Sønderkær, Julie S Bødker, Maria Andersen, Søren Nielsen, Ilse Christiansen, Olav J Bergmann, Martin Bøgsted, Karen Dybkær, Mogens Vyberg, Hans E Johnsen.**
Oral mucosa tissue gene expression profiling before, during, and after radiation therapy for tonsil squamous cell carcinoma. Submitted to PlosOne September 2017.

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Molecular characterization of mucosa in patients with acute myeloid leukemia. Draft included.

ABBREVIATIONS

5-FU	5-fluorouracil
AML	Acute myeloid leukemia
BMI	Body mass index
BSA	Body surface area, an indicator for metabolic mass in the body calculated using e.g. Du Bois formula: BSA = 0.007184 x $Weight^{0.425}$ x $Height^{0.725}$
CD	Cluster of differentiation (cell surface target for immune typing)
CON	Healthy age and gender matched individuals included in the study
COX	Cyclooxygenase
CTCAE	Common Terminology Criteria for Adverse Events of The National Cancer Institute (NCI) of the National Institutes of Health (NIH)
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DRYD	Dihydropyrimidine dehydrogenase
ECOG	Eastern cooperative oncology group performance status
EGFR	Epithelial growth factor receptor
FDG-PET/CT	Flour-Deoxy-Glucose-Positron-Emissions-Tomography/CT scan
FDR	False discovery rate
FFPE	Formalin fixed, paraffin embedded
GCSF	Granulocyte-colony stimulating factor
GGE	Global gene expression
GM	Gastro-intestinal mucositis
H&E	Hematoxylin eosin stain
HER-2	Human epidermal growth factor
HNC	Head and neck cancer
HSCT	High dose chemotherapy supported with stem cell transplantation
HLA	Human leucocyte antigen
IL	Interleukin

IMRT	Intensity modulated radiation therapy
ISOO	International Society of Oral Oncology
JNK	Jun-N-terminal
MASCC	Multinational Association of Supportive Care in Cancer
MAPK	Mitogen activated protein kinase
MHC	Major Histocompatibility Complex
MM	Multiple myeloma
MMP	Matrix metalloproteinase
MNC	Mononuclear cells
MOMP	Mitochondrial outer membrane permeability
mRNA	Messenger-RNA
NCI	National Cancer Institute
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK-cells	Natural killer cells
NM	None/mild mucositis
NSCLC	Non-small cell lung cancer
OM	Oral mucositis
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RT	Radiation treatment
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA
TKI	Tyrosine kinase inhibitor
TNFα	Tumor necrosis factor alpha
TSCC	Tonsil squamous cell carcinoma
UM	Ulcerative mucositis
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

PREFACE

Mucositis of the oral and gastro-intestinal mucosa is one of the most painful side effects of cancer treatment. Clinically it is defined as a state of mucosal inflammation with characteristics that allow graduation in accordance with objective signs and subjective claims, including erythema, ulcerations, pain, nausea and diarrhea. The severely affected patients spend prolonged time in hospital.

Knowledge of the molecular reactions that underlie mucositis has evolved primarily from animal models, based on the assumption that mucositis is one entity clinically and independent of disease and patients characteristics. In these models, potential preventive interventions were tested with promising results; however, only few interventions and no predictive tests were translated into clinical practice.

A systematic review of the literature (Manuscript I) exposed only a limited number of human studies and even fewer including a correlation to clinical mucositis, indicated the need to link clinical data with molecular events in the epithelium. Therefore, we proposed a simple model to conduct a number of comparable scientific pilot studies in three different cancer treatment regimens known to induce mucositis.

The working hypothesis of this study was that molecular analysis of consecutive human mucosa biopsies and peripheral blood samples would reveal molecular mechanisms of importance to our understanding of the mucositis pathogenesis. We aimed to answer following specific scientific questions:

- Was the study program and model feasible?
- Could we identify specific molecular changes in human mucosa and blood samples over time during cancer treatment?
- Were these changes correlated to mucositis severity?

- Could we identify disease and/or treatment specific gene expression patterns?

We correlated the gene expression profiles of consecutive mucosa tissue and blood cell samples before, during, and after treatment with the grade of clinical mucositis among small groups of patients with multiple myeloma (MM), acute myeloid leukemia (AML), and tonsil squamous cell carcinoma (TSCC). The results of these studies are reported in Manuscripts II-IV and yielded the following main results:

- The study was feasible with sufficient tissue quality and no clinical complications observed.
- In response to therapy, we found genes altered in the mucosa tissues dominated by DNA damage, DNA defense and repair in all three groups with no identified correlation to grade of clinical mucositis.
- Differences among the groups were apparent; e.g., early immune regulation was seen only in MM and TSCC but not in AML mucosa.
- In MM, pretreatment expression of the genes *HLA-DR1* and *HLA-DRB5* were potential predictive protective biomarkers for ulcerative mucositis.
- In TSCC, pretreatment expression of the gene *LY6G6C* was a potential predictive protective biomarker for ulcerative mucositis.
- A principal component analysis of the global dataset on mucosal gene expression revealed that patients cluster according to disease indicating that disease is foremost contributor to the variation in gene expression of the mucosa samples and not mucositis grade.
- In brief, this pilot study presents a feasible model and preliminary results that allow us to continue the search for disease specific predictive mucosa gene signatures (MUGS).

DANSK RESUME

Mukositis i mundslimhinden og i mave- tarmkanalen, er en af de mest smertefulde bivirkninger ved kræftbehandling. Mukositis ses klinisk udtrykt i varierende sværhedsgrad hos patienter, der modtager sammenlignelig behandling. De patienter som rammes i svær grad oplever udbredt sårdannelse i mund og svælg, opportunistiske infektioner, opkastning og diarre og indlæggelsestiden for disse patienter øges.

Viden om de molekylære reaktioner i mucosa under kræftbehandling er primært udledt fra dyre eksperimentelle studier ud fra den formodning, at mukositis er *klinisk* varierende og udelukkende afhængig af behandlingsregime. Lovende fund fra disse studier kunne ikke overføres til klinikken og der findes fortsat ingen forebyggende behandling eller prædiktive tests for patienter med svær mukositis.

Baseret på et systematisk litteraturstudie (Manuskript I), som belyste, at der kun er udført få studier om de molekylære reaktioner i human mukosa under kræftbehandling, afdækkede vi et behov for at sammenholde de molekylære reaktioner i vævet med det kliniske udtryk af mucositis. Vi foreslog derfor en simpel model af sammenlignelige pilotstudier i tre forskellige kræft behandlingsregimer, hvor mucosa og blod fra patienter blev analyseret.

Derfor blev arbejdshypotesen for dette studie at molekylær analyse af slimhindebiopsier og blodceller før, under og efter kræftbehandling ville afsløre sammenhænge, der kunne bidrage til vores forståelse af mukositis patogenesen. Vi søgte at besvare følgende spørgsmål:

- Er det muligt at foretage konsekutive biopsier med acceptabelt væv til molekylær analyser uden komplikationer?
- Kan vi identificere specifikke molekylære ændringer i mucosa og blod over tid hos patienter i kræftbehandling?
- Er disse ændringer korreleret til graden af klinisk mukositis?

- Findes sygdoms eller behandlings specifikke biomarkører?

Herefter gennemførte vi tre parallelle kliniske studier med indsamling af mucosa biopsier og blodprøver fra patienter før, under og efter kræftbehandling. Vi inkluderede patienter med henholdsvis knoglemarvskræft (myelomatose, MM), akut myeloid leukæmi (AML) og tonsilkræft (TSCC). Vi udførte gen ekspressions analyse af mucosa og blod og korrelerede resultaterne til graden af klinisk mucositis. Resultaterne er rapporteret i Manuskript II-IV:

- Den opstillede forsøgsmodel gav sufficient væv til genanalyse og var uden kliniske komplikationer.
- Som reaktion på behandling, fandt vi opregulering af gener relateret til apoptose, DNA skade og reparation, i alle tre behandlingsgrupper. Der var også forskelle mellem grupperne; f.eks. sås tidlig involvering af immunsystemet kun i TSCC og MM gruppen.
- Vi kunne ikke identificere tilsvarende ændringer i blodet.
- Disse op- og nedregulerede gener var uafhængig af mucositis grad.
- Vi fandt at generne *HLA-DRB1* og *HLA-DRB5* var potentielle prædiktive biomarkører for svær mucositis hos patienter med MM.
- Vi fandt at genet *LY6G6C* var en potentiel prædiktive biomarkør for svær mucositis hos TSCC.
- En principal komponent analyse af det samlede datasæt for genekspressions i mukosa viste at patienterne clustrer omkring sygdom, der således bidrager med den største variation i ekspression mere end mucositis grad.
- Kort fortalt, dette studie viser en brugbar model til i fremtiden at identificere sygdoms specifikke prædiktive mukositis genekspressions signaturer (MUGS).

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BACKGROUND

1.1. CLINICAL MUCOSITIS

Mucositis is an acute and potentially dose limiting side effect of cancer treatment, and present clinically as inflammation of the oral and gastro-intestinal mucosa (1,2). Generally, 2-3 days following the administration of chemotherapy, gastro-intestinal mucositis (GM) may present as abdominal bloating, vomiting, diarrhea and/or constipation (3,4) and after 7-10 days oral mucositis (OM) may present as erythema and/or ulcers of the oral mucosa (Fig 1) (5–8). The inflammation resolves spontaneously one to two weeks after termination of treatment (9,10). Radiation therapy (RT) induced mucositis is dose dependent, restricted to the radiation field and appear after a cumulative dose of 30-35 grey; concomitant chemotherapy aggravates the condition (11–13). Targeted therapy also induces mucositis. The oral affections resemble aphtous lesions and may be accompanied by a skin rash, but gastro-intestinal symptoms are more common in this group (14–16).

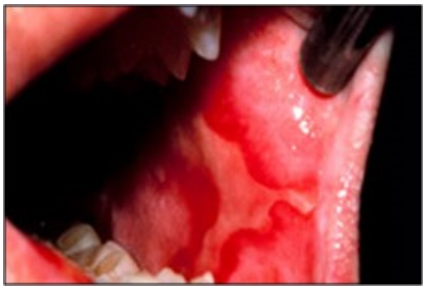

Fig 1. The clinical presentation of severe/ulcerative oral mucositis: confluent painful patches of ulcers impair oral food intake and parenteral feeding may become necessary. Ulcers are the entrance of bacteria and fungi that may cause potential lethal infections. From Sonis 2004 (17).

Cancer therapy also affects the microflora by reducing the diversity and load, which may lead to opportunistic infections and reactivation of latent viruses (18–24).

Mucositis is unevenly expressed among patients and the clinical manifestations have been described in several studies (5,6,14,25). Compared to mild mucositis, severe mucositis is associated with mucosal ulcers, fever, diarrhea, nausea, vomiting, and opportunistic infections. Patients with severe mucositis generally require prolonged hospitalization, need stronger pain relief, feeding tube installation, and intensive care (25–28). Consequently, severe mucositis continues to be a considerable burden to patients and to the healthcare system (29).

The evaluation of clinical oral mucositis is standardized across cancer treatment regimens (30). The anatomical location and the visual presentation of the mucosa (erythema, ulceration) combined with registration subjective symptoms (pain, mouth dryness, inability to eat solid food) is summed up in a scale. The most widely used is oral mucositis toxicity scale (range 0-4) of The World Health Organization (WHO) (Appendix A, Table 1). A graduation of 0-1 indicate no/mild mucositis (NM), whereas at ratings of 2-4, termed ulcerative mucositis (UM), patients are gradually unable to swallow food and may need parenteral feeding. The Common Terminology Criteria for Adverse Events (CTCAE) issued by the National Cancer Institute (NCI) is available for GM scoring on a five level scale (Appendix A, Table 2). At graduations of 1 to 2 vomiting raises from one to two episodes in 24 hours to three to five episodes. Grade 3 indicates more than 6 episodes, whereas grades 4 to 5 are life-threatening gradually leading to death (31).

1.2. INCIDENCE AND RISK FACTORS

The incidence of mucositis is closely related to drug regimen (14,27,32–37), and the strongest mucositis risk factor is the type of cancer treatment (38). Among patients with head and neck cancer (HNC) that receive combined chemo-radiation therapy the incidence is 85%, even with the implementation of intensity modulated radiation therapy (IMRT) (2,39,40). Although radiation treatment (RT) is generally performed as an out-patient treatment, hospitalization is needed in 37%, and a feeding tube is indicated in 51% of these patients (2,26). Among patients with

hematologic cancers, mucositis is generally frequent, and of the patients in high dose chemotherapy receiving autologous stem cell transplantation (HSCT) 80% are affected to some degree, although only 40% severely (27). Among patients with solid tumors in conventional chemotherapy the overall risk is 5 to 40% (41). In patients that receive targeted therapy mucositis is also frequent, however, skin rash and gastro-intestinal symptoms are more common (14,42,43). Mucositis was reported in 66% of patients receiving the anti-mTOR agent Rapamycin (44) whereas skin rash and diarrhea was reported in up to 80% in patients treated with the tyrosine kinase inhibitor (TKI) Erlotinib (45). A combination of different regimens as well as targeted agents seem to have an additive effect (15,46,47) and children generally have a higher risk of developing mucositis compared to adults (33).

Patient-related risk factors include increased dose per kilogram body weight (low body mass index (BMI)), female gender and baseline eastern cooperative oncology group performance status (ECOG) including reduced renal function (27,48). The susceptibility to mucositis expressed in both female gender and children relates to the body surface area (BSA), an indicator of metabolic body mass, from which the dosage of a chemotherapeutic drug is estimated (men 1.9; female 1.6; child 10 yrs. 1.1). Low BSA/bodyweight is positively related to mucositis (5).

A genetic component also seem to be associated with UM. Patients with the autoimmune disease psoriasis are 70% less prone to severe mucositis (1,49). Genomic polymorphisms in genes encoding drug-metabolizing enzymes are associated with higher risk of severe mucositis. For example, certain single nucleotide polymorphisms (SNP) of dihydropyrimidine dehydrogenase (DPYD) that degrades Capecitabine to 5-Fluorouracil (5-FU) may predict severe mucositis. The topoisomerase inhibitor Irinotecan cause severe neutropenia and diarrhea in patients expressing less of the UGT1A1*28 allele. Furthermore, polymorphisms in genes encoding the proteins p53 or MDM2 in patients with advanced non-small cell lung cancer (NSCLC) treated with platinum-based chemotherapy which is now a

U.S. Food and Drug Administration (FDA) approved predictor of severe irinotecan toxicity, although controversy still exists (50–56).

DNA extracted from the saliva of 216 patients suffering various hematologic malignancies and treated with HSCT, was examined for SNPs associated with mucositis severity, and a Bayesian network was built This network could predict severe mucositis with a predictive validity of 81.2% (57). A second similar study included 972 patients with multiple myeloma in HSCT treatment, and eleven SNPs located near matrix metalloproteinase (MMP) 13 was associated with UM (58).

1.3. RECOMMENDATIONS OF CARE

Based on a systematic literature review, The Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO) regularly issue Clinical Practice Guidelines for oral and gastro-intestinal mucositis (59). First, there is a general suggestion in favor of using an oral care protocol for the prevention of oral mucositis across all cancer treatment regimens. Other recommendations are treatment specific; 30 min of oral cryotherapy prevent oral mucositis in patients receiving 5-FU bolus; benzydamine mouthwash prevent oral mucositis in patients with HNC receiving moderate doses RT (less than 50 Gy), without concomitant chemotherapy. Although not reducing the incidence of mucositis, treatment of pain is mandatory. For HSCT patients self-administered morphine for pain relief is recommended, and transdermal fentanyl at levels of 50 μg/h is suggested (60). For patients receiving chemo-radiation for HNC, a 2% morphine mouthwash for reliving for OM is suggested (60). Finally, patients in cancer treatment receive a broad spectrum of antibiotics, antifungal, and/or anti-viral prescriptions to prevent opportunistic infections (39,60–63).

1.4. MOLECULAR BIOLOGY OF MUCOSITIS

The current model of mucositis pathology describe how chemotherapy and/or RT initially inflict DNA damage on the endothelium and the rapidly dividing cells in the basal layer of the epithelium (64–67). Intrinsic apoptotic pathways are up-regulated by reactive oxygen species (ROS), pro-apoptotic regulators BAX/BAK and p53 (68–71). Simultaneously, chemotherapy and/or RT trigger pro-inflammatory cytokines, tumor necrosis factor alpha (TNFα), interleukin (IL) 1-beta, and IL-6 to enter the circulation (6,72–74), that lead to the activation of an inflammatory response via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). This process damage the molecular parts of the submucosa (75). TNFα activates the extrinsic apoptotic pathway via mitogen activated protein kinase (MAPK), Ceramide, and SAPK/Jun (76–78). NF-κB activated COX-2 facilitates prostaglandin productions, while TNFα activates NF-κB and c-JUN in a feedback mechanism leading to apoptosis. Inflammatory infiltration and bacterial colonization lead to further tissue damage, which involves among others MMP, which was recently identified as a key regulator of mucositis (79–81). Barrier function and mucosal integrity is compromised through disruption of tight junctions (82,83). COX-2 initiate angiogenesis and macrophage recruitment down-regulates the inflammatory response (84,85); the production of new tissue results in healing.

Studies reporting global gene expression (GGE) analysis on mucositis were introduced in animal models (86). Following irradiation (35 Gy applied once to the cheek pouch of hamsters), tissue was secured after one, 4, 8 and 24 hours, 5 and 10 days. Within 8 hours, 10 genes related to acute tissue damage were identified. Among these *MAPK*, *Hsp70*, *KRT14* and *SPRR8* were up-regulated. Neither NF-κB nor TNFα were altered before day 10. In a mouse model, 15 Gy was applied to the snout, and the tissues were analyzed before, at day 1, 3, 5, 7, 10, 14, 21 and 28 (87). Generally, the expression of IL-6, IL-1β, TNFα, C-X-C motif ligand 1 (CXCL1) and C-C motif ligand 2 (CCL2) gradually increased until day 7 and rapidly decreased hereafter. Suppression of p53 was found in another radiation mouse

model (83). Most recently, the importance of the epithelial mast cell was examined in a rat model (88). Pathways of anti-inflammatory signaling were up-regulated in the immune competent rats, seemingly protecting against radiation injury whereas the incompetent rats were injured more seriously.

In a model of Irinotecan-treated rats, biopsies from different parts of the gastro-intestinal tract at various time points, were analyzed (0 to 72 hours). More than 500 genes were temporarily altered (76,89,90); among these, an early response of the genes involved in stress response, apoptosis, cell cycle, and transcription. The most dominant pathways were the MAPK, cell cycle, keratinocyte differentiation, B-cell receptor, and apoptotic signaling pathways. The inflammatory pathways NF-κB, Jun, Il-6, TNFα and Bax were up-regulated early. This study also showed that similar pathways were activated in the different anatomic regions of the oral and gastro-intestinal canal. In a recent study on transgenic mice receiving 100 mg/kg 5-FU, gene expression analysis of the intestinal mucosa also revealed a central role of NF-κB (91). In a mice model using Doxorubicin in different doses, apoptosis was observed within one day (92). In addition, the expression of caspase and TCF-4 (a WNT-signaling pathway transcription factor) increased whereas bone morphogen protein (BMP) 4 decreased. Both TCF-4 and BMP4 are involved in the regulation of stem cell proliferation and homeostasis in the epithelial-mesenchymal compartment.

1.5. CELLULAR STRESS, APOPTOSIS AND TARGETS FOR CYTOTOXIC AGENTS

Most conventional chemotherapeutic drugs, radiation and some targeted therapy agents activate the apoptotic pathways through DNA damage (68,93–96). Apoptosis is a genetically determined process of programmed cell death and is a part of normal development and elimination of damaged and unusable cells and of pathological conditions (97). Two classical signaling pathways induce apoptosis (Fig 2). The intrinsic apoptotic pathway is activated by stress factors and physical

or chemical injuries, i.e., hypoxia, radiation, heat shock, aggregation of misfolded proteins or disruption of the cytoskeleton (98). ROS released through mitochondrial outer membrane permeability (MOMP) into the cytoplasm activates a cascade of pro-apoptotic factors, e.g., BCL2 family, p53 and BAX/BAK. The extrinsic or receptor-mediated apoptotic pathway is initiated by an external death ligands of the TNF family (e.g. TNFα, FasL, and TRAIL) situated in the outer cell membrane. These ligands stimulate death receptors e.g., TNFR1, FasR-alfa DR3. Both apoptotic pathways eventually activate caspase, an apoptosis initiating protease. Cytotoxic T lymphocytes and natural killer cells (NK-cells) may activate a third pathway in which granzyme A and B activate procaspase.

Among the therapies that induce apoptosis are radiation, the alkylating agents (e.g., melphalan, bulsulfan, cisplatin), antimicrotubule agents (e.g., vincristine, vinblastine), anti-metabolites (e.g., methotrexate, fluorouracil, cytarabine), topoisomerase inhibitors (irinotecan, etoposide), cytotoxic antibiotics (doxorubicin and daunorubicin) and some targeted agents (e.g. bortezomib, trastuzumab) (46,99–101). Other targeted therapies (monoclonal antibodies and TKI inhibitors) act through the inhibition or blocking of specific molecular targets, e.g., human epidermal growth factor (HER-2), epithelial growth factor receptor (EGFR), or vascular endothelial growth factor (VEGF) (14,15,42).

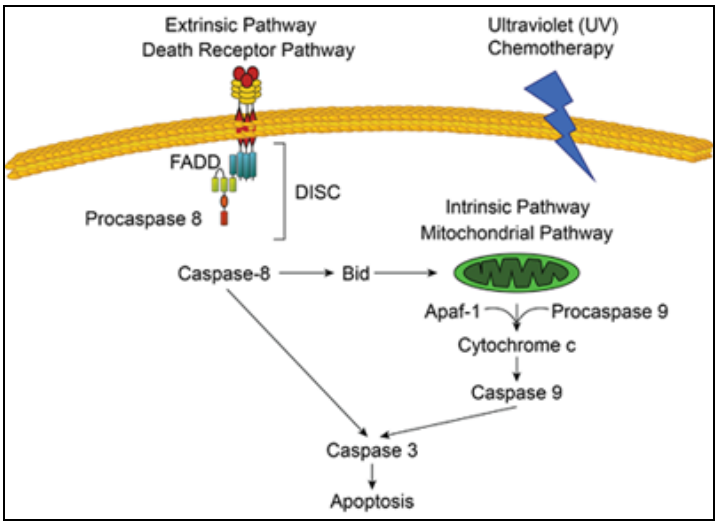

Fig 2. Apoptotic pathways. The intrinsic apoptotic pathway is activated by stress factors and physical or chemical injuries, i.e., hypoxia, radiation, heat shock, aggregation of misfolded proteins or disruption of the cytoskeleton. The extrinsic or receptor-mediated apoptotic pathway is initiated by an external death ligand of the tumor necrosis factor (TNF) family (e.g. TNFα, FasL, and TRAIL). Both pathways activate the final apoptosis inducer caspase3.

1.6. ANATOMY OF THE ORAL MUCOSA

Mucosa is a stratified layer of squamous cells either keratinized or non-keratinized. Compared to other tissues of the body the turnover rate of the continuously proliferating epithelial cells is high, estimated 4-5 days, compared to 39 days for normal skin (102,103). From the basal stem cell layer asymmetric division is followed by symmetric division and amplification and finally post mitotic differentiation (104–107) (Fig 3).

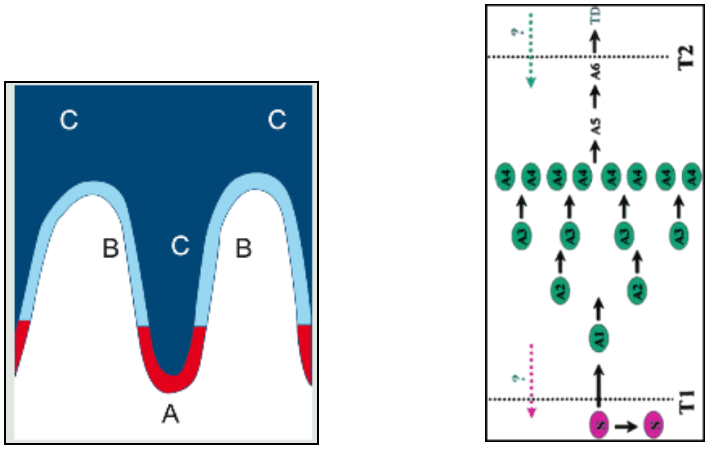

Fig 3. A schematic drawing of normal mucosa anatomy. Left: The white area indicates the submucosa/connective tissue compartment consisting of fibroblasts, muscle, glands, vessels and nerve fibers. A: Stem cell compartment of the basal layer for continuous differentiation (positive for keratin 15 and 19). B: Early differentiated epithelial cells (positive for keratin 6 and 16). C: Supra basal layer of keratinized or non-keratinized cells. From Dabelsteen 2006 (104)*. Right: The basal stem cell layer consists of three cell compartments: asymmetric division in the stem cell compartment (S); division in the amplifying compartment (A) and post-mitotic differentiation (TD). From Tudor et al 2004* (106)*.*

Mucosa is a part of the external barrier of the body that is constantly exposed to microorganisms. The integrity of the epithelium is maintained by epithelial cells tight junctions and a local immune system of migratory dendritic cells (DC) (108,109). DC´s are antigen-presenting cells that monitor changes in oral micro-flora and communicate with T-lymphocytes of the immune system (110,111). DC´s express HLA-DR (Human leukocyte antigen (HLA) of the Major Histocompatibility Complex (MHC)) on the surface (Fig 4). Furthermore, epithelial cells of the gastro-intestinal mucosa express HLA-DR (112,113), but the concentration of DCs is considerably higher in the buccal mucosa than in other regions (110).

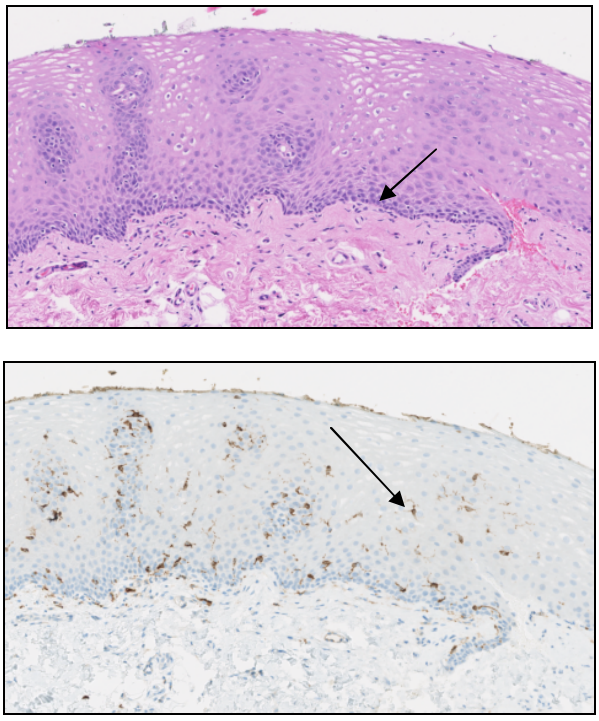

Fig 4. Normal human mucosa. Upper: Hematoxylin-eosin (H&E) stained buccal mucosa (x20). A basal stem cell membrane (arrow) divides submucosa and epithelia. Lower: HLA-DRB5 stained buccal mucosa (x20). The arrow indicate a HLA-DRB5 positive cell with dendritic expansions If present, the DC´s are located in the epithelia and the submucosa close to the basal membrane.

PAPER I: A SYSTEMATIC REVIEW OF MOLECULAR RESPONSES TO CANCER THERAPY IN NORMAL HUMAN MUCOSA

2.1. OBJECTIVES

The objective of this study was to provide a summary of previous studies on the molecular changes in normal human mucosa during cancer therapy. Our impression was that the current model of mucositis pathogenesis was based primarily on animal studies (6,80,86,89,114–116); results that were challenging to translate into a clinical care (117).

2.2. METHODS

We performed a systematic review in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (118). We constructed a search strategy based on a combination of medical subject headings (MeSH), EMTREE headings and natural language terms to search in MEDLINE and Ovid Embase (Appendix B). Clinical studies that described molecular changes in the mucosa of patients in cancer treatment at risk of developing mucositis, were included (119).

2.3. PAPERS RETRIEVED FROM THE SYSTEMATIC REVIEW

Seventeen eligible full-text articles were extracted for evaluation and included in the analysis; nine papers describing chemotherapy affected mucosa (6,65,67,68,120–124) (Appendix C, Table 1); and eight papers on radiated mucosa

(75,125–131) (Appendix C, Table 2). We did not identify any papers on targeted therapy.

2.4. CONCLUSIONS

Only two of the studies (one paper on archived unspecific mucosa tissue and one paper including three AML patients) applied the GGE analysis. The studies on both chemotherapy and RT affected mucosa exposed that apoptosis and involvement of inflammatory mediators were generally present. For the chemotherapy group, increased endothelial permeability, through tight junction disruption was involved. For the RT group, reestablishment of the epithelial proliferation through cytokeratin production were indicators of early defense mechanisms. However, of the retrieved papers, only two studies correlated the described molecular events in the tissues to the clinical expression of mucositis (124,130); low expression of thymidylate synthase, that control DNA replication and is targeted by the chemotherapeutic agent 5-FU and was associated with UM (124). Among patients with various HNC tumors the number of 27E10 positive macrophages in the submucosa was correlated to the grade of mucositis. However, because of the heterogeneity among the studies meta-analysis was not possible.

2.5. A MODEL FOR MUCOSITIS RESEARCH IN A HUMANS

Based on our findings, we suggested conducting a number of pilot studies of similar nature to the ones retrieved, but with comparable designs, similar conditions and with a link from the molecular events in the epithelium to the clinical expression of mucositis. The aim of this approach was to reveal the molecular pathways associated with mucositis phenotypes. The design would include serial human mucosa biopsies and blood from patients in different cancer treatment regimens at risk of developing mucositis. The methodologies applied would align with the concept of “precision medicine” as proposed by the National Research Council (132,133).

CLINICAL STUDY: MATERIALS AND METHODS

3.1. STUDY DESIGN

We planned to obtain three consecutive buccal biopsies and blood tests before, during and after therapy from each 10 patients in three different cancer treatment regimens: patients with multiple myeloma (MM), patients with acute myeloid leukemia (AML), and patients with tonsil squamous cell carcinoma (TSCC) along with one buccal biopsy and one blood test from 10 healthy individuals (CON). A plan is showed in Table 1. A detailed description of the timeline of tissue sampling in each cohort, appear as figures in the chapters of the individual papers.

Table 1. Study design of clinical study

Tissue samples	MM	AML	TSCC	CON
1. biopsy and peripheral blood	Before melphalan (day0)	Before first induction therapy (day0)	Before RT (day0)	one biopsy one blood sample
2. biopsy peripheral blood	Two days after melphalan (day2)	Three days after initiation of induction therapy (day2)	After one week of RT (day7)	X
3. biopsy and peripheral blood	Three weeks after melphalan (day21)	Before second induction therapy (day21)	Twenty days after the last RT session (day21)	X

3.2. STRENGTHS AND LIMITATIONS

The primary limitation of this study, was the number of consecutive biopsies that one patient provided. However, this restriction is even more relevant using animal tissue, since animals are sacrificed at sampling (86,87). Second, during active cancer treatment, the patients are at risk of attracting complicating infections, especially after breaking the mucosal barrier. However, among the human studies retrieved in the review, no patient related complications were reported. Third, how did we decide when to harvest the tissues to provide relevant information? Before any visible macroscopic damage, the process that lead to mucositis is triggered immediately upon initiation of cancer therapy. This has been documented previously (8,134). Therefore, in order not to compromise the neutropenic patient, we took the second biopsy before onset of clinical mucositis and neutropenia. We wanted to avoid harvesting disintegrated tissue dominated by inflammatory mediators and to gain insight of the cellular processes that underlie the inflammatory state. Finally, our method would provide only a snapshot of an ongoing process, equaling the time points that we decided; however, we did not aim to give the full picture and we are aware that this study is a pilot-set up that would potentially disclose associations that could guide future larger and more specific studies.

Also relevant is a discussion on whether to use e.g., buccal swap biopsies; both DNA and mRNA can be extracted from this less invasive methods (135). The cells gained from this technique consists of desquamated keratinocytes (136) and is an easy source of DNA extraction. However, the processes in the submucosa or immune related alterations will not appear when examining mRNA from these cells as most dendritic cells migrate to and from the epithelium (110,137). Furthermore, morphological information would not be available.

3.3. PATIENTS INCLUDED IN THE STUDY

The Committee on Health Research Ethics of Northern Denmark approved the clinical protocol (ref. N-20100022). We recruited patients at Aalborg University Hospital from September 1st 2010 to April 30th 2013. Patients were enrolled if at age 18 or above, if cancer treatment naïve and if they were without uncontrolled competitive diseases. We obtained informed written consent in accordance with the Declaration of Helsinki.

3.3.1. PATIENTS WITH MULTIPLE MYELOMA

MM is a blood cancer characterized by malignant transformation of plasma cells (138). High-dose melphalan, supported with autologous stem cell transplantation (HSCT), has been the standard treatment for decades (139). Ten included patients with MM received a standard treatment: initial chemotherapy entailed Cyclophosphamide 500mg/m^2 i.v. day 1 and 8; Velcade 1,3 mg/m2 i.v. day 1, 4, 8, and 11; and Dexamethasone 20mg p.o. day 1-2, 4-5, 8-9, 11-12, repeated in 3 to 4 series. Before harvest of stem cells, the patients were primed with Cyclophosphamide 2 g/m^2 and treated with recombinant granulocyte stimulating factor (G-CSF). CD34+ hematopoietic stem cells were harvested by leukapheresis and vital frozen in liquid nitrogen. Two days after administration of Melphalan (200mg/m^2), stem cells were re-infused.

3.3.2. PATIENTS WITH TONSIL SQUAMOUS CELL CARCINOMA

TSCC is a localized epithelial cancer of the tonsil. We included eight patients who had histology-verified TSCC and a metastasis-negative Flour-Deoxy-Glucose-Positron-Emissions-Tomography/CT (FDG-PET/CT) scan (140). All patients received curative intended intensity modulated RT on six weekly fractions of 2 Gy according to international guidelines in the Danish Association of Head and Neck Cancer 2004 protocol (141,142). Dependent of the patient´s age, general health

status and tumor staging according to the TNM system (143), RT was supplied if indicated with concomitant cisplatin (40mg/m^2) once a week during RT (144).

3.3.3. PATIENTS WITH ACUTE MYELOID LEUKEMIA

AML is a blood cell cancer of the myeloid lineage characterized by accumulation in the marrow of abnormal blasts that interfere with normal hematopoiesis and infiltrate the blood with immature blasts (138,145,146). We included six patients with de novo diagnosed AML. Treatment consisted basically of Cytarabine (100mg/m^2) and Daunorubicin (60mg/m^2) for 5 respectively 2 days if age 70 or above and if age less than 70, for 10 respectively 3 days, supplied if indicated with Etoposide (100mg/m^2). The treatment was adjusted to age and general health status according to protocol.

3.3.4. HEALTHY INDIVIDUALS

We planned to recruit ten medically healthy, non-smoking, age and gender matched individuals at the Department of Maxillofacial Surgery during other planed benign surgery (third molar removal before orthognathic surgery). However, the majority of these patients did not meet the age matching criteria and recruitment expanded to medically healthy non-smoking age and gender matched department employees and friends. After informed consent, we took out one biopsy and one blood test.

3.4. COLLECTION OF DATA AND HANDLING OF SAMPLES

3.4.1. CLINICAL DATA AND ASSESSMENT OF MUCOSITIS

All patients underwent initial evaluation including medical history and clinical examination at the study entry. We screened the patients for dental infections and these were removed prior to treatment.

Trained nurses recorded the OM grade daily on MM and AML during hospital stay. TSCC received ambulant treatment and were evaluated for OM status weekly during RT until the acute stages of mucositis disappeared. Data of gastro-intestinal mucositis from the MM group was retrieved retrospectively through records.

3.4.2. MUCOSA BIOPSIES

According to the study plan, the biopsies were harvested from the buccal mucosa. After thorough mouth rinse with chlorhexidine and application of local anesthesia, 0,5ml citanest (felypressin/prilocain 30 mg/ml + 0,54mikg/ml; DENTSPLY, York, PA, US), a lens formed 5mm biopsy was taken with a scalpel approximately 1cm inferior to the parotid papilla. The wound was tightly sutured with resorbable Vicryl 4.0 (Ethicon, Summerville, NJ, US). We instructed the patients to rinse with chlorhexidine twice daily until removal of sutures after 10 days. The one-half of the biopsy was immediately embedded in RNA-later (Ambion, Thermofischer Scientific, Waltham, MA, US) for 24 h; then, it was frozen at -80^{o}C. The other half was fixated in 10% neutral-buffered formalin, and shortly after embedded in paraffin and kept until further analysis. All samples were successively stored in the biobank until all material was secured.

3.4.3. BLOOD SAMPLES

On the same day as the biopsy, fifteen ml of EDTA mixed venous full blood was taken. Mononuclear cells (MNC) were isolated using the in-house standard purification protocol (available at http://miltenyibiotec.com) following the manufactures guidelines for the Ficoll-Paque^{TM} (GE Healthcare, Little Chalfont, Buckinghamshire, UK), density gradient centrifugation and a Leukosep^{R}Tube (Greiner Bio-One GmbH, Frickenhausen, Germany). Cells were stored in a freezing medium containing 10% dimethyl sulfoxide in units of 5mio, vital frozen at -196^{o}C in liquid nitrogen. All samples were successively stored in the biobank until all material was secured.

3.5 DATA ANALYSES

3.5.1 GLOBAL GENE EXPRESSION ANALYSES

Genetic information stored in the DNA is translated to protein via messenger-RNA (mRNA) transcription, illustrated in Fig 5. The gene expression technology offers a genome wide approach to this central cellular process (147). We performed the analysis at the mRNA level, to provide a print of transcriptional activity in the tissues (phenotype).

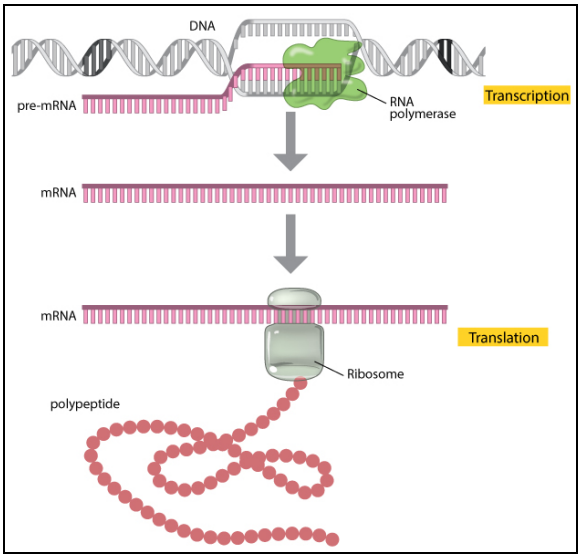

Fig 5. DNA transcription and translation. Information stored in the DNA is translated to protein via mRNA transcription. http://www.nature.com/scitable/topicpage/translation-dna-to-mrna-to-protein-393.

We used the Affymetrix GeneChip Human Exon 1.0 ST Arrays with approximately 4 probes per exon and 40 probes per gene. With more than 1.4 million probe sets, analysis of both gene expression and alternative splicing was available. The workflow is shown in Fig 6. The mucosa samples were homogenized using TRIzol^{R} Reagent (Invitrogen, Paisley, UK) and total RNA was isolated using mirVana^{TM}

miRNA Isolation Kit (Ambion^{R}/Invitrogen, Paisley, UK) according to manufactures protocol (Life Technologies Corporation). RNA amplification was performed on TP Basic Thermocycler real time PCR instrument (Biometra^{R}) following standard reaction conditions as described in the manufacturers manual "The Ambion^{R}WT Expression Kit" (Applied Biosystems^{R}) starting out with 100ng total RNA. The Quality of RNA product vas evaluated by NanoDrop and Bioanalyzer using Agilent RNA 6000 Nano Kit (Agilent Technologies^{R}). The samples were prepared for hybridization to Affymetrix GeneChip Human Exon 1.0 ST Arrays using Affymetrix GeneChip WT Terminal Labeling and Controls Kit (P/N 901524), following the manufacturer's instructions. CEL-files were generated by Affymetrix GeneChip Command Console Software. A similar procedure was applied on MNC from blood samples from the MM and TSCC group.

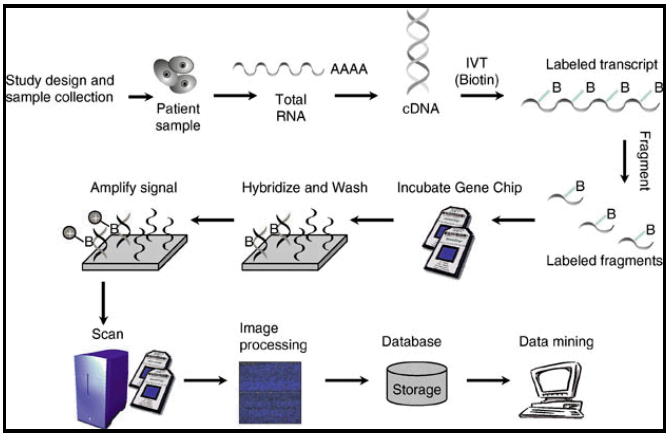

Fig 6. The workflow of gene expression analysis.

3.5.2 IMMUNOHISTOCHEMISTRY

The formalin fixed, paraffin embedded (FFPE) tissue-blocs were cut at 4µm and mounted on glass sheets, dried for 24 hours and hereafter kept frozen (-20 ^{o}C) until the dying procedure. One slice of all tissue samples were H&E stained for control.

Based on the results from the gene expression analysis, antibodies were acquired for immunohistochemical stain. After evaluation using an in-house optimized protocol, tissues were stained accordingly. The specimens were then scanned in a Hamamatsu Nanozoomer slide scanner and analyzed in the NDP viewer software.

3.6 STATISTICAL ANALYSES

All statistical analyses were performed with R (148) version 3.2.0 and Bioconductor packages (149).

3.6.1 ESTIMATION OF POWER SIZE

To detect genes that varied more than two-fold between test points with a false discovery rate (FDR) of less than 0.05% and a power of 90%, we applied the method described by Lee and Whitmore (150), implemented in the R-package, size-power (Qui 2008) (151). Ten patients in each group was calculated to be sufficient for detecting major significant differences.

3.6.2 DATA PROCESSING

The Affymetrix Expression Console produced CEL files that were preprocessed and summarized at the gene level using the RMA algorithm with the Bioconductor package affy using custom CDF-files (152). The preprocessing of the CEL files resulted in the expression levels of 38,830 genes for each array and was annotated with Ensembl gene identifiers (ENSG identifiers). Patient CON09 was included in the normalizations of the gene expression data in the MM group, but excluded in the statistical analysis because he suffered from the autoimmune disease psoriasis. CON09 was excluded completely from the TSCC and the AML cohorts.

3.6.3 DETECTION OF DIFFERENTIAL EXPRESSION

With patient ID as a cluster variable, we used the linear model for microarray data (limma package in R), a mixed linear model, and an empirical Bayes approach to test for significant differences in gene expression levels between day2 and day0,

and between day21 and day0 (153). We performed an unpaired test with the limma package to test for significant differences in gene expression between patients on day0 and controls. We adjusted the p-values for false discovery rates and they were controlled with the method described by Benjamini-Hochberg (154), for each test. We considered adjusted p-values below 0.05 as significant. According to their mucositis experience, the patients were divided into UM or NM.

We applied the Mann-Whitney test to test for the relationship between mucositis severity and duration of neutropenia, leukopenia, and thrombocytopenia in the MM group. We also used the Mann-Whitney test to evaluate differences between groups in the numbers of in-hospital days and years of progression free survival (PFS) in the MM and AML group.

The GGE data set of all nine CON samples and eight TSCC samples was divided into subsets by gene biotypes: protein coding, pseudogene, miRNA, rRNA, snoRNA, snRNA, linRNA, and antisense transcript. Each dataset was subjected to hierarchical clustering using Pearson correlation as a distance measure and average linkage as the algorithm method. Using adjusted p-values from the pairwise test, all genes were ranked by the degree of differential expression (DDE) calculated as: DDE = -log10 (P-value) * (ABS(FC)/FC), where the fold change (FC) in gene expression between the groups was compared. This approach leaves highly up-regulated genes at the top of the ranked list and downregulated genes at the bottom. Each ranked list was subjected to gene set enrichment analysis using the GSEA software and Reactome pathways as gene sets (155–157). Gene sets with an FDR < 0.05 were considered enriched.

For the detection of alternative spliced genes the CEL-files produced by the Affymetrix Expression Console were imported as full exon import file into and analyzed by Partek Genomic Suite software following manufacturers default workflow (Partek Incorporated, St. Louis, USA).

PAPER II: MOLECULAR CHARACTERISTICS OF HIGH-DOSE MELPHALAN ASSOCIATED MUCOSITIS IN PATIENTS WITH MULTIPLE MYELOMA: A GENE EXPRESSION STUDY ON HUMAN MUCOSA

Below, Fig 7 outline a detailed study plan for the MM group.

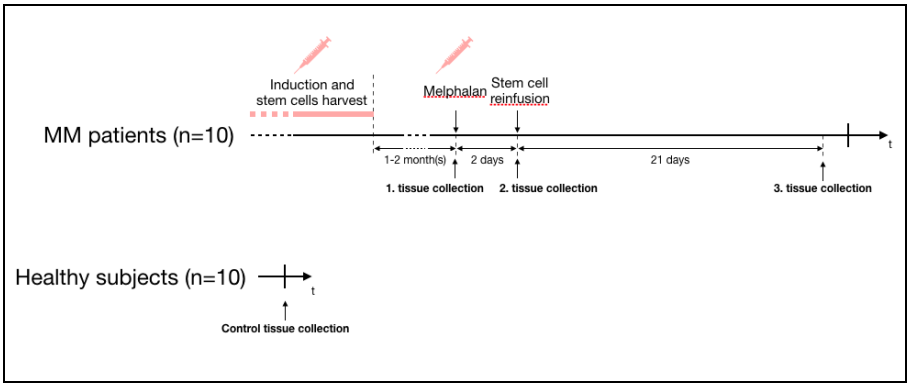

Fig 7. Study design for the MM cohort. Tissue was collected before Melphalan, 2 days after melphalan at stem cell reinfusion and at an outpatient control visit 21 days after melphalan. Patients without progressive disease were recruited and clinical data was collected after the induction treatment. Tissues were collected and stored successively in our biobank and analyzed collectively at the same laboratory.

In this study of patients with MM receiving melphalan, we identified the up- or down-regulation of genes belonging to pathways that were previously recognized as inducers of mucositis, e.g., apoptotic, inflammatory, and DNA repair genes.

However, these changes were not associated with the severity of clinical mucositis (Fig 8).

Furthermore, we identified both inducers and inhibitors of apoptosis. Melphalan induces oxidative stress and upregulates apoptosis-related genes (78,158). In our material, *EDA2R*, an inducer of apoptosis, was up-regulated. *EDA2R* encodes a TNF-receptor that mediates the NF-κB and JNK pathways resulting in caspase induced apoptosis (75,123,159,160). However, *INPP5D*, which encodes a membrane protein that negatively regulates JNK signaling, and limits Fas-FasL-induced apoptosis in T-lymphocytes found at mucosal surfaces was also up-regulated (161). Furthermore, we found alteration of five genes involved in suppression of the p53 apoptotic pathway: *MDM2*, *CUL9*, *E2F7*, and *TIGAR* (up-regulated) and *SERRPINB10* (downregulated). *MDM2* encodes a protein ligase that inhibits p53-mediated cell cycle arrest and apoptosis (162). Several studies have reported p53 as an inducer of mucositis (67–69). However, the genes related to apoptosis in our study, including *EDA2R,* did not correlate to the level of clinical mucositis and was also up-regulated in patients who did not develop clinical mucositis. A similar pattern was seen in the expression of *POLH*, a gene that encodes a specialized polymerase that accurately replicates damaged DNA.

TREM2 and *LAMP3* (up-regulated) encodes membrane proteins expressed on DC´s and involved in T-cell activation and inflammation (163,164). The protein encoded by *TREM2*, can bind and phagocytose yeast species, Gram-positive and Gram-negative bacteria (165,166). Additional, DC´s secrete cytokines (e.g., IL-12 and type I interferon) in response to antigen exposure. IL-12 mobilizes natural killer (NK) cells. Also, we saw *NCR3LG1* up-regulated, a gene encoding a ligand triggering NK cells (167). Thinning of the epithelium, causing exposure of the microbiota in combination with changes in its composition and concentration was previously recognized to contribute to the development of mucositis (19,168). Our results confirmed this; however, we did not find an association to mucositis severity.

The genes *ABCA12* and *CEL* were up-regulated on day2. *ABCA12* encodes a membrane transporter protein primarily involved in the keratinocyte lipid-barrier that maintains homeostasis in the epidermis (169). *ABCA12* has not previously been associated with mucositis, but *ABCA12* may be a similar barrier protection. *CEL* encodes a lipase with multiple functions in lipid metabolism, and is expressed in macrophages (170). The expression of both these genes was also without relation to the clinical expression of clinical mucositis

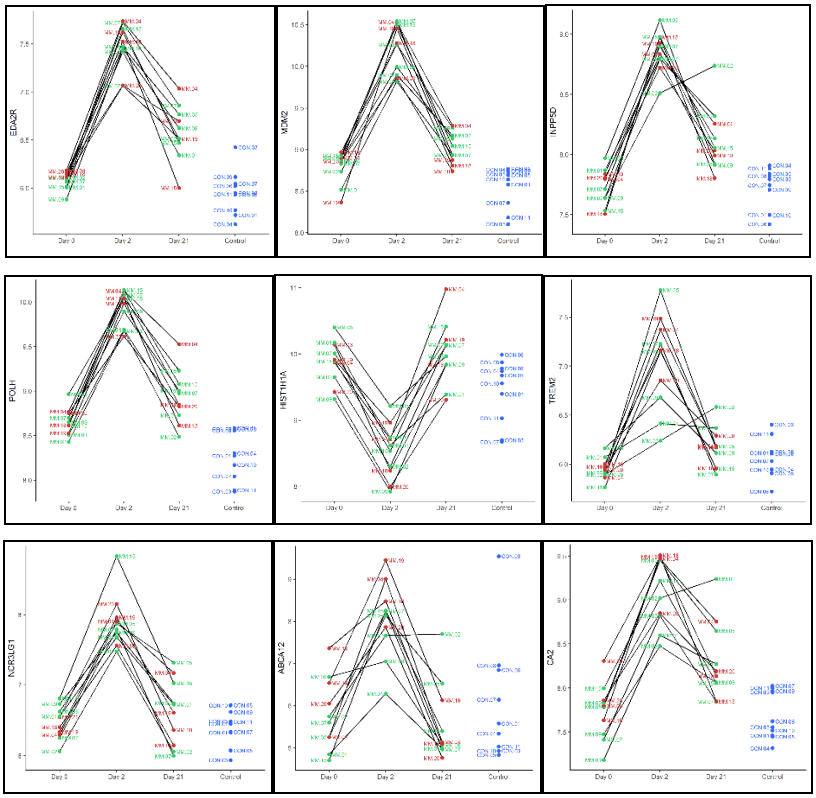

Fig 8. Dot plot of gene expression before, day 2, and day 21 after melphalan. Red = UM; green = NM; blue = CON. First row: genes associated to apoptosis: EDA2R, MDM2, and INPP5D. Second row left: genes affecting DNA repair/transcription, POLH and HIST1H1A. Second row right and third row left: genes related to inflammation, TREM2 and NCR3LG. Third row right: genes related to metabolism: ABCA12 and CA2. Mucositis severity was not correlated to the alterations of these genes.

We identified potential predictive biomarkers for mucositis severity: *HLA-DRB1* and *HLA-DRB5*. These genes belong to the MHC Class II family members and encode a surface protein located on specialized antigen presenting cells, e.g., Langerhans cells (112,171–173). Both genes were up-regulated in NM compared to UM and CON (Fig 9).

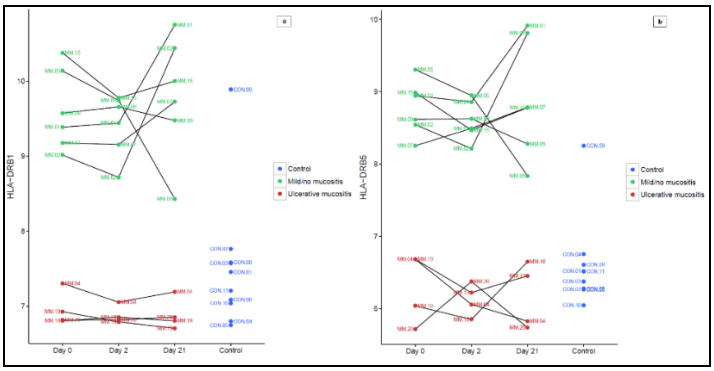

Fig 9. Genes differentially expressed according to mucositis grade. Left: HLA-DRB1 and right: HLA-DRB5 at baseline (day0), two days (day2), and 21 days (day21) following high-dose melphalan. The level of expression of these genes did not vary upon treatment. Red = UM; green = NM; blue = CON.

HLA-DRB1 was expressed in two splice variants: NM_002124 (UM and CON) containing six exons and NM_001243965 (NM and CON09) containing seven exons (Fig 10).

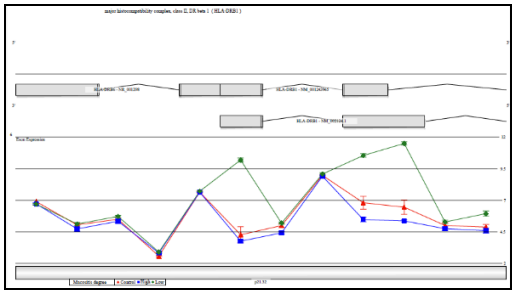

Fig 10. Alternative splicing analysis of HLA-DRB1. HLA-DRB1 was expressed in two splice variants: NM_002124 (six exons), and NM_001243965 (seven exons). NM and CON09 expressed the longer variant.

We confirmed the result from the gene expression analysis with immunohistochemical stain for HLA-DRB5 (Fig 11). Generally, if present, the HLA-DRB5 positive cells were localized in the lower part of the epithelium, near the basal membrane, around the papillae, and in the upper part of the submucosa. The morphology of the positively stained cells was similar to DC´s.

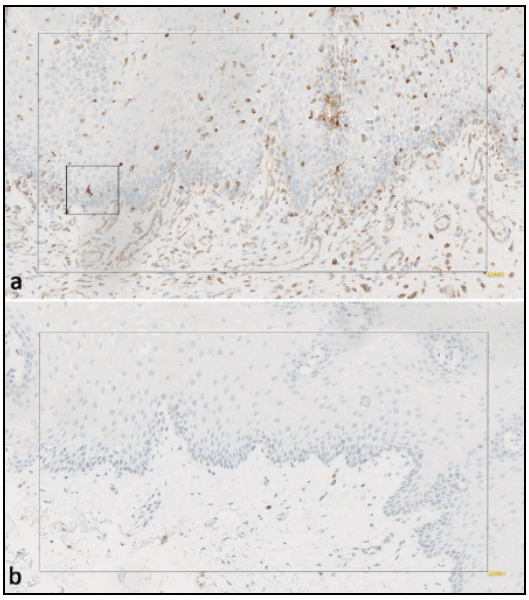

Fig 11. Immunohistochemical analysis for HLA-DRB5 of the oral buccal mucosa (×20 magnification) from patients with multiple myeloma. (a) High HLA-DRB5 expression in MM01 with mild mucositis. (b) Low HLA-DRB5 expression in MM18 with severe mucositis. The square highlight the morphology of one of the HLA-DRB5 stained cells: noticeable cellular extensions similar to those observed in dendritic cells.

CON09 expressed the same elevated levels of *HLA-DRB1* and *HLA-DRB5* as the NM. This patient suffered from psoriasis. Psoriasis is a skin disease of auto-immune origin, characterized by reduced apoptosis; polymorphisms in HLA-related genes were previously reported (177). One study described that these patients are 70% less prone to develop mucositis (8,49). CON09 also expressed ABCA12 at different level together with NM in contrast to UM and the other CON´s. This was also found in a previous gene expression study on psoriasis patients (178).

PAPER III: ORAL MUCOSA TISSUE GENE EXPRESSION PROFILING BEFORE, DURING, AND AFTER RADIATION THERAPY FOR TONSIL SQUAMOUS CELL CARCINOMA

Below the study design for the TSCC cohort is shown (Figure 12).

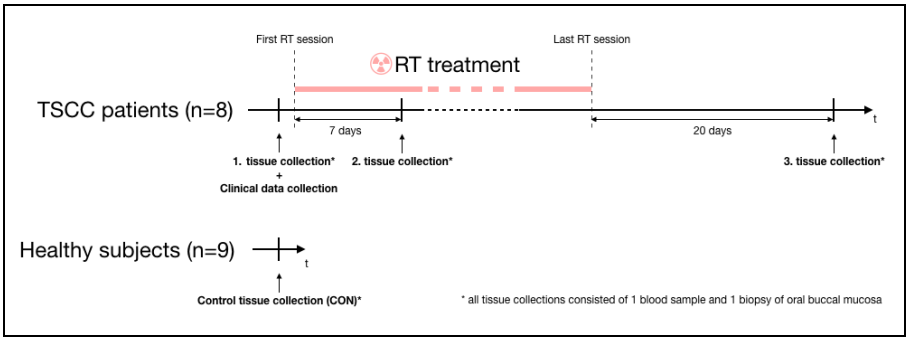

Fig 12. Study design for the TSCC cohort. Tissue was collected before RT, after one week of RT and 20 days after the last RT session. Clinical data was collected at entrance to the study. Tissues were stored successively in our biobank and subsequently analyzed collectively at the same laboratory.

Upon seven days of RT, we identified genes related to apoptosis that were up-regulated in patients with TSCC, similar to the response seen in melphalan treated patients with MM: *EDA2R* that encodes a TNF receptor activating the NF-κB and jun-N-terminal (JNK) apoptotic pathways, and *MDM2* encoding a ligase that inhibits p53-mediated apoptosis (162). Indicating additional DNA damage, six members of the histone cluster families (e.g., *HIST1H3B)* were downregulated. Histones are basic nuclear proteins responsible for nucleosome structure. A previous study described histone down-regulation in response to RT in cell lines

(174). *POLH* was up-regulated like in the MM group indicating DNA repair. Transcriptional activity was also affected; *KRT16* (up-regulated) encodes keratin16, an epithelial filament protein that is responsible for cell structure that is expressed in early differentiated epithelial cells (106,125,175). Also, keratin16 may participate in innate immunity regulation in response to mucosal trauma (126,175). Contrary, *MKI67* encoding the proliferation marker Ki-67 was downregulated. In a previous study Ki-67 was up-regulated two weeks after radiation (126). Dot-plots of selected altered genes is shown in Fig 13. In long-term in response to RT, we found alterations of *SCIN* (down–regulated) that encodes a protein with regulatory functions in exocytosis (176). We expected to find this gene expressed in the salivary glands, however immunohistochemical stain revealed that scinderin was expressed in the epithelial cells (Fig 14). Finally, *MIR31HG*, a long non-coding snRNA with unknown function, was only long-term up-regulated in patients receiving cisplatin. None of these alterations were correlated to mucositis severity (Fig 13).

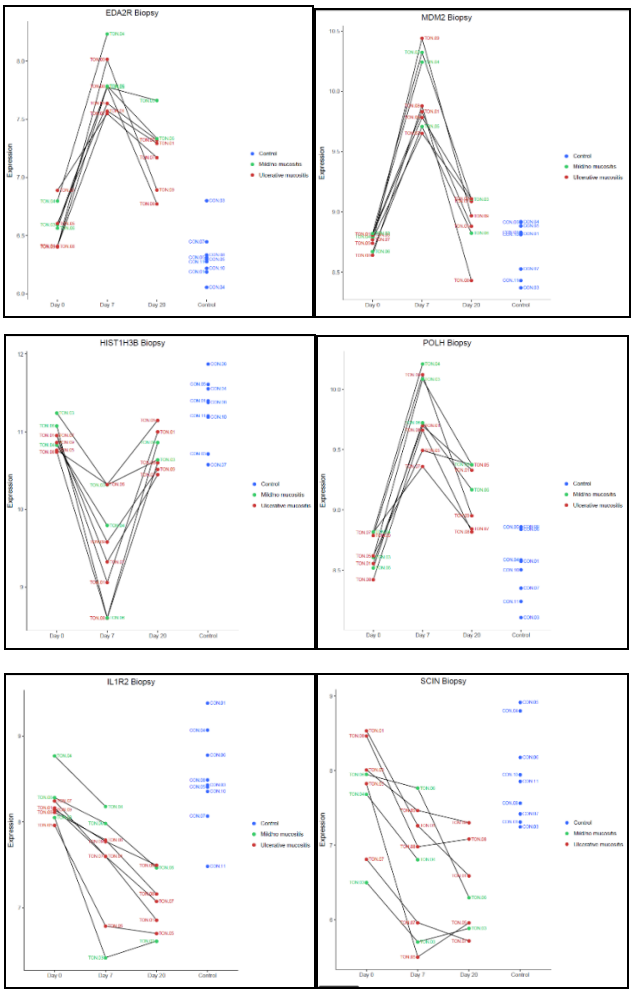

Fig 13. Gene expression before (day0), after 7 days of RT (day7), and 21 days (day21) following the total RT dosage administrated. Red = UM; green = NM; blue = CON. First row: genes associated with apoptosis: EDA2R and MDM2. Second row: genes affecting DNA repair/transcription, HIST1H3B and POLH. Third row: genes altered long-term: IL1R1 and SCIN. These genes were independently expressed of clinical mucositis.

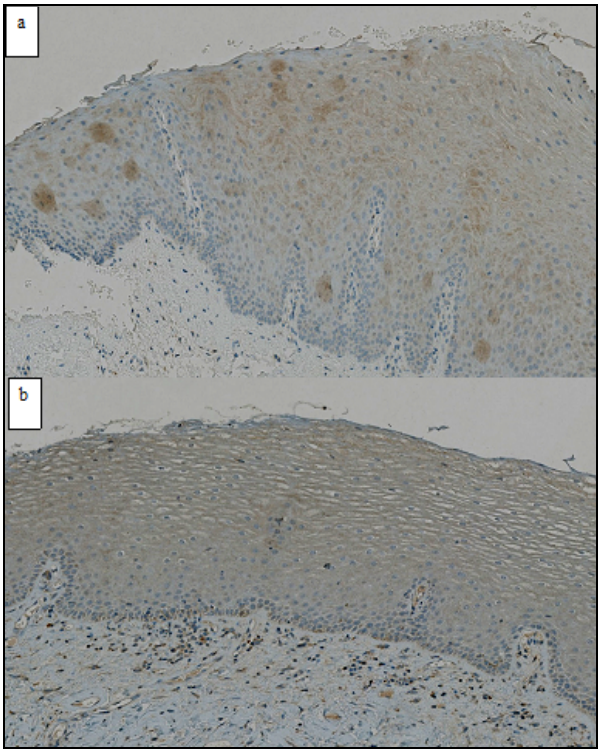

Fig 14. Immunohistochemical analysis of buccal oral mucosa (x15 magnification) stained for scinderin shows a staining of the epithelial cells. (a) High scinderin expression in the mucosa of patient CON05. (b) Low scinderin expression in the mucosa of patient TSCC07, 20 days after the last RT session.

Although not statistically significant, *LY6G6C* (lymphocyte antigen 6 complex, locus G6C) was up-regulated (x3.78; P=0.0995) in patients with NM compared to UM before treatment (Fig 15). *LY6G6C* belongs to a cluster of leukocyte antigen-6 genes of the MHC Class III, encoding a cell signaling surface protein (177).

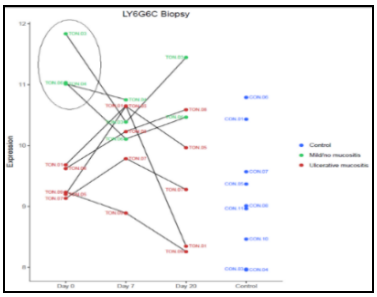

Fig 15. LY6G6C expression in the mucosa of patients with TSCC receiving RT at baseline (day0), after seven days of RT (day7), and 21 days (day21) after the last RT session. Red = UM; green = NM; blue = CON. Patients with NM encircled at baseline.

Before RT application, we found altered genes in the mucosa of TSCC compared to CON: *LIFR* (leukemia inhibitor factor alpha), *PDGFRA* (platelet-derived growth factor receptor alpha), and *SPARCL* (secreted protein acidic and cysteine rich) (Fig 16).

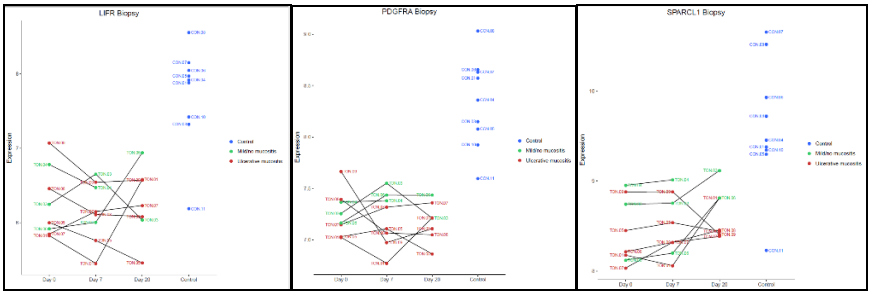

Fig 16. Dot plots of LIFR, PDGRF, and SPARCL expression in the mucosa of patients with TSCC receiving RT at baseline (day0), after seven days of RT (day7), and 20 days (day20) after the last RT session. Red = UM; green = NM; blue = CON. All three genes are expressed unaffected of RT.

These genes were independently expressed of alcohol consumption, smoking habits, and p16 overexpression in the tumor. The gene *LIFR* encodes a transmembrane receptor protein of the type 1 cytokine receptor family, which is involved in cellular differentiation, proliferation, and survival and acts as an

inhibitor of the p53 apoptotic pathway. Low expression was identified as both a suppressor and a promotor of carcinogenesis (178). *PDGFR* encodes a cell-surface tyrosine kinase receptor for the platelet-derived growth factor family members that activates cell migration and chemotaxis pathways in wound healing (179); certain mutations in the *PDGFRA* gene was identified in cancer progress (180). *SPARCL* is involved in extracellular matrix synthesis and was downregulated in a number human cancer types (181). It remains unclear why these genes connected to carcinogenesis were expressed in clinically normal appearing oral mucosa at a distance from the tonsil squamous cell carcinoma.

In the blood, we also identified a gene signature before any treatment was applied. Fig 17 shows two of 29 altered genes, *RNU6-620P* (downregulated; FC=11.8; P=5.80e-80) and *RNU6-622P* (up-regulated; FC=7.3; P=8.62e-05) compared to normal controls. This was a finding without association to mucositis, however interesting, and a potential candidate for cancer diagnosis.

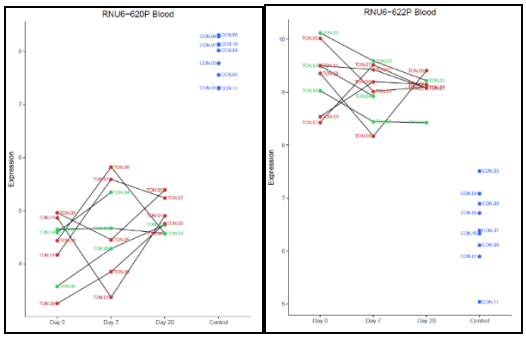

Fig 17. Dot plots of RNU6-620Pand RNU6-622P expression in blood of patients with TSCC receiving RT at baseline (day0), after seven days of RT (day7), and 20 days (day20) after the last RT session. Red = UM; green = NM; blue = CON. Both genes are expressed independently of RT.

PAPER IV: MOLECULAR CHARACTERIZATION OF MUCOSA IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

Below Fig 18 shows a detailed study plan for the AML group.

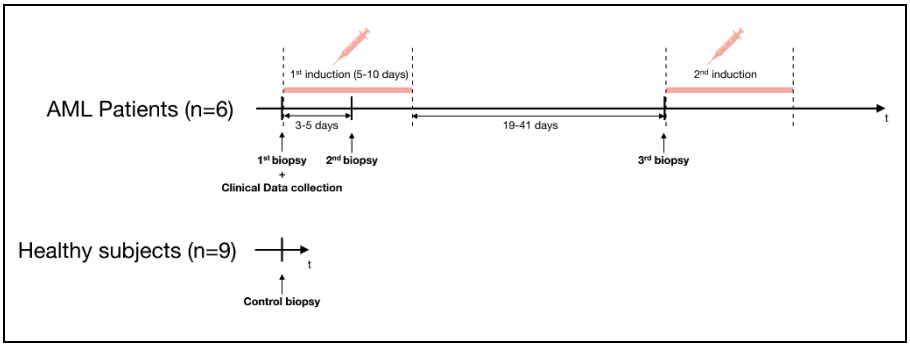

Fig 18. A detailed description of the study design for the AML cohort. The first biopsy was collected before initiating induction therapy (Day0); the second after 3-5 days of chemotherapy (Day3); and the third biopsy was harvested at an out-patient control visit 19-41 days after the last chemotherapy session (Day27), just before initiating the second induction treatment. Tissues were stored successively in our biobank and subsequently analyzed collectively in our laboratory.

The clinical characteristics and demographics of the patients with AML is shown in Table 2 and data during treatment in Table 3. Clinical mucositis was registered with an average score of 1.3 (range 0-3); two patients experienced UM. One AML responded completely to the cancer treatment, while five relapsed. Of these, only one survived. Overall survival was 2.7 years (range 1.5-4.3); for the UM group 3.9 years (range 3.4-4.3) and for the NM group 1.9 (range 1.5-4.2). Event free survival was 1.1 (range 0-4.0) for the UM group 2.9 (1.5-4.3) and for the NM group 0.3 (range 0-1).

Table 2. Patients characteristics and demography at entrance to the study

Patient	Age	Gender	ECOG[a]	BMI[b]	Smo[c]	Alc[d]	FAB[e]	Treatment
AML01	58	m	1	29.3	0	0	M6	Cy 10 days Da 3 times Mylo
AML02	69	m	1	26.8	1	0	M2	Cy 8 days Da 3 times Etop 4 days
AML04	59	f	1	21.4	0	0	M5	Cy 8 days Da 3 times Etop 4 days Mylo
AML05	58	f	1	23.8	0	0	M4	Cy 10 days Da 3 times Etop 5 days
AML07	75	f	1	27.1	0	0	M2	Cy 5 days Da 2 times
AML09	74	m	2	23.9	1	0	M4	Cy 5 days Da 2 times

Abbreviations: a=Eastern Cooperative Oncology Group performance status; b=body mass index; c=smoking more than 10 cigarettes per day; d=drinking more than 21 units of alcohol weekly; e=FAB: French-American-British subtype classification; Cy=Cytarabine 100mg/m^2 twice daily; Da=Daunorubicin 50mg/m^2 once every second day; Etop= Etoposide 100mg/m2 once daily. Mylo= Mylotarg 3mg/m^2 once;

Table 3. Patients clinical data during chemotherapy

	[a]Mucositis grade	[b]Time b2	[c]Time b3	Relapse	[d]Overall survival	[e]Event free survival	Status at follow up
Patients with ulcerative mucositis							
AML01	3	3	41	MDS AML	3.4	1.5	dead
AML02	3	4	19	no	4.3	4.3	CR
Patients with no/mild mucositis							
AML04	0	4	No third biopsy	AML	4.2	1.0	relapse
AML05	0	3	22	AML	1.5	0	dead
AML07	1	3	27	AML	1.5	0	dead
AML09	1	5	27	AML	1.5	0	dead

Abbreviations: a= Mucositis estimated according to WHO (REF Quinn); b=number of days from initiation of induction therapy to second biopsy; c= number of days from end of first induction therapy to third biopsy. The third biopsy was secured immediately before initiation of second induction treatment. d=overall survival estimated as years from diagnosis/enrolment into study until death; c=event-free survival estimated as years from diagnosis/enrolment into study to disease progression/relapse. CR=complete response

In response to treatment, we identified a total of four genes differently expressed (Appendix F, Fig 19): two genes of the histone cluster family, *HIST1H1A* and *HIST1H2BM*, were downregulated, *POLH* encoding a transcriptional DNA directed polymerase and *NOTCH1* encoding a membrane protein responsible for intercellular signaling that regulates interactions between physically adjacent cells, were up-regulated.

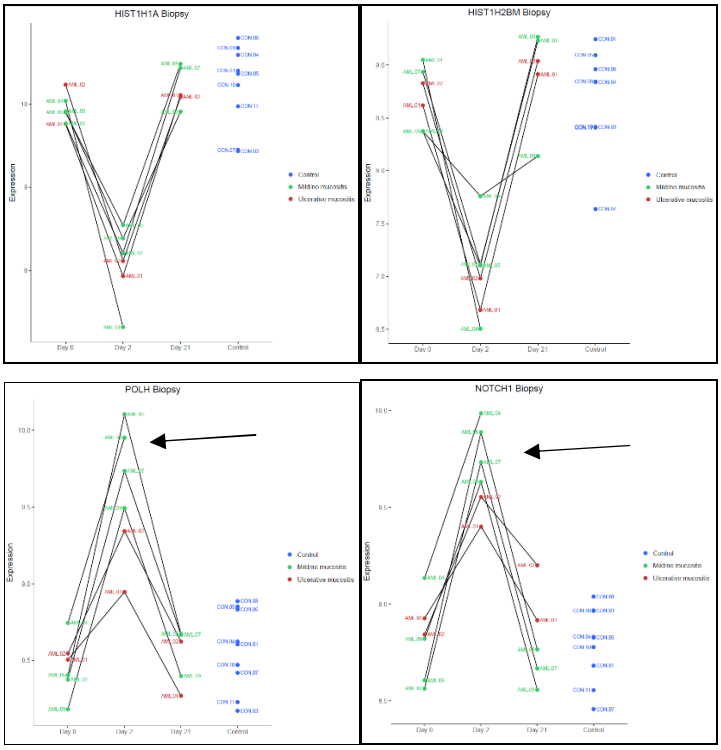

Fig 19 Gene expression before (Day0), after 3-4 days of chemotherapy (Day3), and before initiation of second induction therapy (Day27). Red = UM; green = NM; blue = CON. First row: genes of the histone family: HIST1H1A and HIST1H2BMB. Second row: POLH and NOTCH1. Although not statistically significant, there was a tendency towards upregulation of POLH and NOTCH1 in response to treatment among the patients that did not develop mucositis, see arrows. These genes were also differently expressed in the MM and TSCC group, but not with the same distinction.

Before treatment was initiated two genes were differently expressed in the mucosa, *LINC01975* and *RNU6-996P* (up-regulated) (Fig 20).

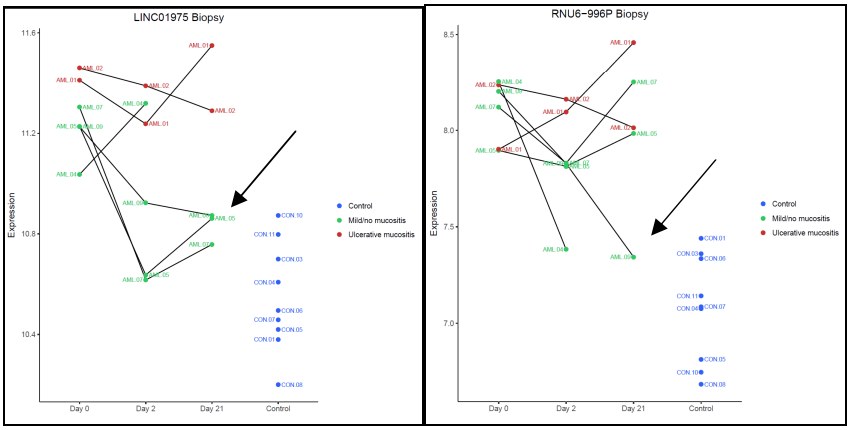

Fig 20. Gene expression of LINC01975 (left) and RNU6-996P (right) *before (Day0), after 3-4 days of chemotherapy (Day3), and before initiation of the second induction therapy (Day27). Red = UM; green = NM; blue = CON. The level of expression seem to normalize in response to treatment in some patients, see arrows.*

DISCUSSION

Initially, mucositis has been assumed to be the result of chemotherapy and RT causing apoptotic and necrotic changes in the cells of the epithelial basal stem cell layer, being more sensitive to genotoxic injury due to a high turnover rate (106,182). Several studies have pointed to initial apoptosis but also to inflammatory mediators as the key inducers of mucositis in animal models (6,70,74,91,183,184), but also in humans (75,89,116,160,185). Among these, TNF-α, the interleukins IL-1β, IL-6, IL-10, the transforming growth factor beta (TGF-β), p53, NF-κB and MMP´s, but with conflicting results. Because of the central role for inflammation, many anti-inflammatory medications has been tried and worked well in the animal models, but did not reduce mucositis; e.g., Pentoxifylline and Thalidomide (TNF-α inhibitors), synthetic prostaglandin and Misoprostol (186–189), or Celecoxib and Infliximab (selective inflammatory inhibitors) (190,191). This leads to discussion weather to use animal models to study mucositis regarding similarities and differences between human and mouse inflammatory reactions. Although debated, some studies have shown a poor correlation, mainly on B-cell receptor signaling, macrophage and monocyte function, and the expression over time of the alpha chain of the HLA-DR class II (192). In addition, in more of the animal studies the mucosa was scratched to provoke oral mucositis to appear, which may have distorted the results (193–195). Our model of harvesting human mucosa tissue for analysis proved feasible and we identified alterations in apoptotic, DNA damage and repair genes changes in the mucosa in response to cancer treatment in all three cohorts. However the treatment induced changes did not correlate to the level of clinical mucositis.

There may be a complexity of factors involved in pathogenesis of mucositis involving both host response, microbiome, treatment modality, type of disease and patient phenotype. Based on identification of both disease and treatment specific differences in gene expression in our study, we propose that the mechanisms

underlying mucositis must be studied using human tissues (192,196). This statement is emphasized by the identification of two a potential prognostic biomarkers, in both cases genes encoding cell surface proteins involved in immune signaling (*HLA-DRB1*, *HLA-DRB5*, and *LY6G6C*). This finding indicate that protective immunity is a central issue in mucositis pathogenesis, but dependent of the patient phenotype. Moreover, among patients with AML upregulation of the two genes *POLH* and *NOTCH1* seem to protect against severe mucositis. Additionally, we merged gene expression data from all three cohorts (MM, TSCC, and AML) and performed a principal components analysis (Fig 20). Patients cluster according to disease indicating that disease is foremost contributor to the variation in gene expression of the mucosa samples and not mucositis grade.

The results in these three pilot studies, leads us conclude that the model of consecutive human biopsies is feasible to design prospective clinical validation trials, including sufficient numbers of patients to characterize molecular mucositis and identify disease specific predictive mucosa gene signatures (MUGS).

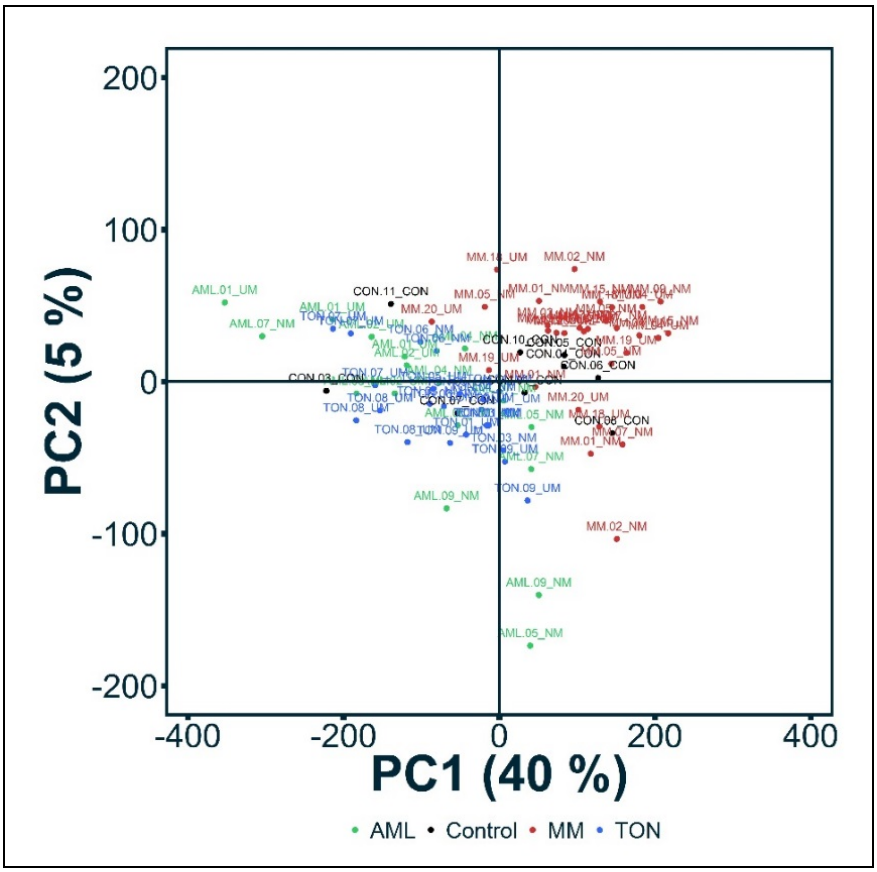

Fig 20. PCA analysis of the gene expression of mucosa biopsies before, during and after treatment from the three cohorts, MM, TSCC, and AML. Red = MM; Green = AML; Blue = TSCC; Black = CON. Patients do not cluster according to mucositis grade, but cluster according to disease.

PERSPECTIVE AND FUTURE RESEARCH

This pilot study has generated new hypotheses to investigate. Most importantly, that the pretreatment phenotype of the local immune system in the mucosa among the MM and the TSCC cohort distinguish patients according to mucositis grade. Since such potential predictive genetic markers were present in the mucosa tissue before treatment in MM and TSCC, a prospective validation trial must involve more patients having only one biopsy taken prior to treatment, which would simplify the study. Furthermore, this approach may allow us to expand the model to study other disease categories, e.g., patients with malignant lymphoma, all patients receiving HSCT or all patients with various head and neck cancers instead of only TSCC. The perspective is, that if we confirm the importance of these biomarkers, we may be able to stratify patients before treatment in the future and adjust treatment including supportive care accordingly.

Finally, we have issued a request for a patent on a potential method to diagnose TSCC in the blood using *RNU6-620P* and *RNU6-622P* as biomarkers (Appendix G). We plan a study recruiting all patients referred to the ENT department on suspicion on HNC in parallel with developing a method using PCR instead of GGE to simplify diagnosis for clinical application.

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First, I owe gratitude to the participating patients, quote: “I know that patients before me had to endure in order for me to receive my treatment today”. Being in the middle of a major life crisis, diagnosed with a potential fatal cancer disease, they donated tissue thereby inflicting themselves additional discomfort during cancer treatment.

My supervisor Hans E. Johnsen was essential for keeping me on track and I am deeply thankful for guidance through my studies.

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- Data manager Charles Vesteghem for providing clear-sighted figures

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FIGURES AND TABLES

CURRICULUM VITAE

This PhD work was initiated at the last year of my specialty training in maxillofacial surgery at the Department of Maxillofacial Surgery in collaboration with the Department of Hematology at Aalborg University Hospital. The subsequent experimental work took place at the Research Laboratory of the Department of Hematology and at the Department of Pathology, Aalborg University Hospital.

After graduating from the School of Dentistry at Copenhagen University, adventure brought me to practice dentistry in Greenland. Following 10 years of "wildlife" in the Arctic, an educational position at Aalborg University Hospital brought me and my family back to Denmark.

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APPENDICES

APPENDIX A: CLINICAL MONITORING OF MUCOSITIS

Table 1: WHO oral mucositis assessments scale	
grade 0	no patient discomfort or erythema only
grade 1	soreness present with or without erythema
grade 2	ulcers present but able to eat solids food
grade 3	ulcers present only able to take liquid diet
grade 4	ulcers present alimentation not possible
Abbreviations: WHO=World health Organization	

Table 2: CTCAE Gastrointestinal disorders	
Grade 1	Increase of <4 stools per day over baseline
Grade 2	Increase of 4-6 stools per day over baseline
Grade 3	Increase of >=7 stools per day over baseline, incontinence
Grade 4	Life threatening, urgent intervention indicated
Grade 5	Death
Abbreviations: CTCAE = Common Terminology Criteria for Adverse Events of The National Cancer Institute (NCI) of the National Institutes of Health (NIH) of gastrointestinal disorders.	

APPENDIX B: LITERATURE SEARCH

Algorithm for electronic search in MEDLINE, chemotherapy and targeted therapy		
1	Mucositis/ or exp stomatitis/	16311
2	(mucosa adj3 inflammation*).ti,ab.	816
3	(mucositis or stomatitis).ti,ab.	21735
4	1 or 2 or 3	33127
5	exp Gene Expression/ or exp Gene Expression Profiling/	477129
6	(expression* or profiling or transcript* or analys* or analyz*).ti,ab.	5886084
7	exp Biomarkers/	787048
8	biomarker*.ti,ab.	162860
9	((salvia or oral or mucosa) adj3 (smear* or sample* or biopsy or biopsies or tissue*)).ti,ab.	17241
10	or/5-9	6391114
11	exp Antineoplastic Agents/	952924
12	Antineoplastic Combined Chemotherapy Protocols/	125973
13	Consolidation Chemotherapy/	308
14	Maintenance Chemotherapy/	1024
15	combined modality therapy/ or chemoradiotherapy/ or chemotherapy, adjuvant/ or neoadjuvant therapy/ or photochemotherapy/ or Molecular Targeted Therapy/	234159
16	(chemotherap* or molecular' target* therap*).ti,ab.	345042
17	or/11-16	1285457
18	exp Neoplasms/	2962050
19	(neomplasm* or cancer).ti,ab.	1350009
20	or/18-19	3291994
21	4 and 10 and 17 and 20	2113
22	21 not (exp animals/ not humans.sh.)	2031
23	limit 22 to "review"	178
24	22 not 23	1853
Algorithm for electronic search in Ovid Embase, chemotherapy and targeted therapy		
1	mucosa inflammation/	28686
2	exp stomatitis/	44129
3	(stomatitis or mucositis).ti,ab,kw.	28421
4	(mucosa adj3 inflammation*).ti,ab,kw.	1262
5	or/1-4	78879

6	exp gene expression/	1345969
7	exp genetic transcription/	864515
8	exp genetic procedures/	1556727
9	(gene* adj3 (expression* or profiling or transcript*)).ti,ab,kw.	594742
10	biological marker/ or exp cell marker/	288243
11	biomarker*.ti,ab,kw.	261887
12	((salvia or oral or mucosa*) adj3 (smear* or sample* or biopsy or biopsies or tissue*)).ti,ab,kw.	31927
13	or/6-12	3195709
14	exp antineoplastic agent/ or molecularly targeted therapy/	1891297
15	exp chemotherapy/	590012
16	(chemotherap* or molecular* target* therap*).ti,ab,kw.	514794
17	or/14-16	2153387
18	exp neoplasm/	4007936
19	(cancer or neoplasm*).ti,ab,kw.	2051085
20	18 or 19	4267080
21	5 and 13 and 17 and 20	2975
22	21 not ((exp animal/ or nonhuman/) not exp human/)	2882
23	limit 22 to "review"	937
24	22 not 23	1945
Algorithm for electronic search in MEDLINE, radiotherapy		
1	Mucositis/ or exp stomatitis/	16536
2	(mucosa adj3 inflammation*).mp.	835
3	(mucositis or stomatitis).mp	30793
4	1 or 2 or 3	36350
5	exp Gene Expression/ or exp Gene Expression Profiling/	549370
6	(expression* or profiling or transcript* or analys* or analyz*).mp	7191673
7	exp Biomarkers/	819103
8	biomarker*.mp.	439680
9	((salvia or oral or mucosa*) adj3 (smear* or sample* or biopsy or biopsies or tissue*)).mp.	26260
10	or/5-9	7636703
11	exp Radiotherapy/	167199
12	exp Radiation/	453417
13	Radiation Injuries/	32276
14	(radiotherap* or radiation* or irradiation).mp.	684168
15	or/11-14	902060

16	exp Neoplasms/	3093726
17	(neoplasm* or cancer).mp.	2972806
18	16 or 17	3486594
19	4 and 10 and 15 and 18	1087
20	19 not (exp animals/ not humans.sh.)	1040
21	limit 20 to "review"	93
22	20 not 21	947
Algorithm for electronic search in Ovid Embase, radiotherapy		
1	exp stomatitis/	43052
2	mucosa inflammation/	27972
3	(stomatitis or mucositis).ti,ab,kw.	27806
4	(mucosa adj3 inflammation*).ti,ab,kw.	1245
5	or/1-4	77064
6	exp gene expression/	1271586
7	exp genetic transcription/	819928
8	exp genetic procedures/	1514592
9	(expression* or profiling or transcript* or analys' or analyz*).ti,ab,kw.	3723339
10	biological marker/ or exp cell marker/	275886
11	biomarker*.ti,ab,kw.	246805
12	((salvia or oral or mucosa*) adj3 (smear* or sample* or biopsy or biopsies or tissue*)).ti,ab,kw.	31231
13	or/6-12	5133909
14	exp radiotherapy/	442944
15	exp radiation/	621562
16	exp radiation injury/	62192
17	(radiotherap* or radiation* or irradiation).ti,ab,kw.	650962
18	or/14-17	1191939
19	5 and 13 and 18	1887
20	exp neoplasm/	3905765
21	(neomplasm* or cancer).ti,ab,kw.	1814752
22	20 or 21	4142376
23	19 and 22	1631
24	23 not ((exp animal/ or nonhuman/) not exp human/)	1593
25	limit 24 to "review"	298
26	23 not 25	1333

Table 1. Characteristics of the included papers, chemotherapy

Paper	No	Cancer type	Treatment	Tissue	HCG	Time from therapy to biopsy	Method of analysis	Mucositis measuring	Molecular or genetic alterations	Conclusions
Wardill 2016	23[1]	Various	Chemotherapy miscellaneous	oral mucosa	7	Day 0 up to +11	IHC	Yes#	claudin-1, ZO-1 occludin IL-1β; IL-6 TNF MMP-2; MMP-9	Increased tissue permeability through tight junction damage. Increase in inflammatory cytokines IL-1β, IL-6, TNF, MMP-2 and MMP-9. No correlation to clinical mucositis.
Mougeot 2013[33]	9	Various	Doxorubicin Cyclophosphamide 5-FU	oral mucosa archive	No	Miscellaneous , less than 30 days	GGE	No	Various	Proof-of-concept study showing extraction of eligible microarray data from FFPE-treated specimens. Alterations seen in pathways of apoptosis, DNA repair, innate immunity, inflammation and bacterial invasion.
Mougeot 2011[34]	3	AML	Cytarabine Daunorubicin	oral mucosa	3	Day 0; +2	GGE	No	ASS1; SLC39A6; CSNK1A1; DUT GBAS HNRPA0; MDM2; TRIAP1 TM7SF3	Pre-treatment AML-specific immune deregulation. Post-treatment inflammatory damage and p53 induced inhibition of apoptosis
Lalla 2010[35]	3	Various	HSCT miscellaneous	oral mucosa	No	Day -10; +10; +28; +100	PCR	Yes*	COX-2; mPGES	Inflammatory markers COX-2 and mPGES peaked at day +10. A correlation between pain scores and mucositis severity. Pilot study.
Logan 2007[36]	18	Various	Chemotherapy miscellaneous	oral mucosa	4	Day 0 up to +11	IHC	Yes#	COX-2; NF-kB	The inflammatory mediators COX-2 and NF-kB were not correlated to severity of clinical mucositis
Gibson 2006	20[1]	Various	Chemotherapy miscellaneous	oral mucosa	4	Day 0 up to +11	TUNEL	Yes#	terminal deoxynucleotidyl transferase (TdT)	Apoptosis occur before day 3 upon chemotherapy administration, starts to decline after 6 days, but has not returned to pre-treatment level at day 11.
Bowen 2005[24]	23[2]	Various	Chemotherapy miscellaneous	duodenal mucosa		Day 0; +1; +3; +5; +16.	IHC	No	p53 Caspase-3 Bax Bak Mcl-1	Apoptotic markers of the Bcl-2 family, Bax/Bak and p53 increased upon initiation of chemotherapy and returned to normal level at day 3, whereas the anti-apoptotic member Mcl-1 decreased.
Santini 2004[40]	50	Colorectal	5-Fluorouracil	colonic mucosa	No	Day 0	IHC	Yes WHO	Thymidylate synthase	Low expression of thymidylate synthase, a key enzyme controlling DNA replication and a target for 5-FU was associated with grade 2-5 mucositis (WHO mucositis assessment scale).
Keefe 2000[39]	23[2]	Various	Chemotherapy miscellaneous	duodenal mucosa		Day 0; +1; +3; +5; +16	TUNEL	No	terminal deoxynucleotidyl transferase (TdT)	TdT a marker of apoptotic activity was seven-fold increased at day one.

Abbreviations: No=number of patients enrolled in the study. 1: Wardil 2016 and Gibson 2006 uses the same patient cohort. 2: Bowen 2005 and Keefe uses the same patient cohort. AML=acute myeloid leukemia. MM=multiple myeloma. HSCT=high dose chemotherapy with autologous stem cell support. 5-FU= 5-fluorouracil. HCG=Healthy Control Group. GGE=global gene expression; IHC=immunohistochemistry; PCR=Polymerase Chain Reaction. OMI=. COX=cyclooxygenase; mPGES.=microsomal prostaglandin E synthase. IL=interleukin. TNF=tumor necrosis factor. NK-kB=nuclear factor kappa-light-chain-enhancer of activated B cells. Bax, Bak. Mcl-1= members of the intrinsic apoptotic pathway Bcl-2 family regulated by p53 activated caspase-3. TUNEL= terminal deoxynucleotidyl transferase dUTP nick end labelling (a method for detecting DNA fragmentation). TdT= Terminal deoxynucleotidyl transferase. FFPE=formalin fixed and paraffin embedded. #Unspecific or no annotation of applied method; * Oral Mucositis Index (OMI) according to Schubert et al 1992. WHO=mucositis assessment according to World Health Organization (Quinn et al 2007)

Table 2. Characteristics of the included papers, radiotherapy

Paper	No	Cancer type	Treatment and dose	Tissue	HCG	Time from therapy to biopsy	Method of analysis	Mucositis measuring	Molecular or genetic alterations	Conclusions
Bonan 2007	10	HNC Mixed	Radiotherapy 46-60 Gy	oral mucosa	7	Before and after 2 weeks	IHC	Yes WHO	CD68 p53 Ki-67	Grade 1 mucositis is associated with increased number of inflammatory CD68 positive cells (macrophages), apoptosis (p53) and the presence of the cell proliferation marker Ki-67.
Bonan 2006	11	HNC Mixed	Radiotherapy 46-80 Gy	oral mucosa	No	Before and after 3 weeks	IHC	Yes WHO	Cytokeratin 1 Cytokeratin 6 Cytokeratin 10 Cytokeratin16	Grade 1 mucositis is associated with increased levels of cytokeratin 1, 6, 10, 14 and 16 compared to non-mucositis areas reflecting a defense towards radiation.
Yeoh 2005	28	Colorectal Mixed	Radiotherapy 25 Gy or 45 Gy/5-FU	colonic mucosa archive	No	Miscellaneous 4-65 days	IHC	Yes#	NF-kB; COX-2	Comparing short term RT with long term RT/5-FU the inflammatory markers NF-kB and COX-2 were equally expressed. Microvascular injury (telangiectasia, fibrosis and sclerosis) was associated with raised levels of NF-kB and COX-2
Dörr 2002	22	HNC	Radiotherapy 12-64Gy	oral mucosa	No	Miscellaneous 0-45 days	[^{3}H]-TdR in-vitro incubation	Yes*	[^{3}H]-TdR	Restoration of epithelial proliferation after initial apoptosis is initiated following the first week of radiation as measured by the expression of [^{3}H]-TdR, a marker of DNA synthesis.
Prott 2002 long term alter..	Ni	SCC HNC	Radiotherapy 60Gy	oral mucosa		Before, at 60Gy and 6-12 months after RT	IHC	No	ICAM-1 (CD54) VCAM-1 (CD106) E-selectin (CD62E) LFA-1 (CD11b/CD18) Mac-1 (CD11a /CD18) VLA-4 (CD49d) 27E10 25F9 RM3/1(CD163)	Subepithelial endothelium (markers CD54,CD106 and E-selectin) and subtypes of leucocytes (markers CD11b/CD18, CD11a /CD18, CD49d, 27E10, 25F9, CD163) were investigated. The expression of CD106 was downregulated after RT, whereas CD11b/CD18 and CD49d positive cells increased. After RT the radiated tissues have a reduced number of vessels and a different pattern of endothelial adhesion proteins as well as macrophages subpopulations.
Handschel 2001a late effect	Ni	SCC HNC	Radiotherapy 60Gy	oral mucosa	No	Before, at 60Gy, and 6 month after	IHC	No	ICAM-1 (CD54) VCAM-1 (CD106) E-selectin (CD62E) LFA-1 (CD11b/CD18) Mac-1 (CD11a /CD18) VLA-4 (CD49d) 27E10 25F9 RM3/1(CD163)	Sub-epithelial endothelium (markers CD54,CD106 and E-selectin) and subtypes of leucocyte (markers CD11a/CD18, CD11b/CD18, CD49d, 27E10, 25F9, CD163) were investigated. RT causes sub-epithelial migration of leucocytes which reverses to normal after cessation of RT. However some migrated cells persist in the tissues 6 month after RT (CD11b/CD18 and CD49d positive cells) A different pattern of adhesion molecules and macrophages subpopulations is are seen after RT.
Handschel 2001b increase	13	SCC HNC	Radiotherapy 60Gy	oral mucosa	No	Before, at 30Gy and 60Gy	IHC	Yes WHO	27E10 25F9 RM3/1(CD163) CD3 CD4 CD8 CD15	Subtypes of macrophages (markers: 27E10, 25F9, CD163), granulocytes (marker CD15) and T-lymphocytes (markers CD3, CD4, CD8) were investigated. Only 27E10 positive macrophages increased upon RT indicating an intermediate inflammatory response. There was a positive correlation between mucositis grade and the number of 27E10 positive macrophages.
Handschel 1999 irradiation	13	SCC HNC	Radiotherapy 60Gy	oral mucosa	No	Before, at 30Gy and 60Gy	IHC	Yes WHO	beta1-integrin (CD29) beta2-integrin (CD18) ICAM-1 (CD54) VCAM-1 (CD106) E-selectin(CD62E)	The transmembrane membrane signaling protein beta2-integrin increased while beta1-ingegrin stayed unchanged upon RT. The endothelial adhesion proteins CD54 and CD62E increased in expression while CD106 remained at low levels.

Abbreviations: No= number of patients enrolled in the study. Ni=not indicated; HNC=head and neck cancer. SCC= squamous cell carcinoma. Gy=Grey; 5-FU=5-fluorouracil; HCG=Healthy Control Group; RT=radiotherapy; IHC=immunohistochemistry; [^{3}H]-TdR= tritiated thymidine; WHO=mucositis assessment according to World Health Organization (Quinn et al 2007); #Unspecific or no annotation of applied method; *RTOG/EORTC scoring system according to the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer

APPENDIX D: GENE LIST MM

Gene symbol	FC	p-value	adjusted p-value	Qualified GO term	Function
Up-regulated genes day2 versus baseline					
MDM2	2.69	2.37e-15	3.07e-11	MDM2 oncogene, E3 ubiquitin protein ligase	Apoptosis
EDA2R	2.63	1.85e-15	3.07e-11	Ectodysplasin A2 receptor	Apoptosis
CUL9	2.25	1.26e-15	3.07e-11	Cullin-9	Apoptosis
INPPD5	2.18	8.39e-14	4.66e-10	Inositol Polyphosphate-5-Phosphatase	Apoptosis
TIGAR	2.17	7.08e-10	8.87e-07	Chromosome 1 open reading frame 5	Apoptosis
E2F7	2.06	2.60e-13	1.05e-09	E2F transcription factor 7	Apoptosis
NCR3LG1	2.70	1.14e-10	1.94e-07	Natural killer cell cytotoxicity receptor 3 ligand 1	Immune response
LAMP3	2.26	4.39e-06	0.0011	lysosomal-associated membrane protein 3	Immune response
TREM2	2.12	2.78e-09	2.92e-11	Triggering receptor expressed on myeloid cells	Immune response
FKBP5	2.04	6.66e-	0.0082	FK506 Binding Protein 5	Immune

		05			response
POLH	2.42	1.60e-14	1.24e-10	Polymerase; DNA directed	Transcription
ARNTL	2.40	2.65e-06	0.00080	Aryl hydrocarbon receptor, nuclear translocator-like	Transcription
NFIL3	2.20	4.29e-05	0.0011	Nuclear factor, interleukin 3 regulated	Transcription
ABCA12	4.73	8.87e-07	0.00034	ATP-binding cassette sub-family A, member 12	Metabolism
CEL	4.64	3.68e-15	3,58e-11	Carboxyl ester lipase	Metabolism
CA2	2.57	9.99e-10	1.18e-06	Carbonic anhydrase II	Metabolism
SLC39A6	2.53	1.16e-10	2.00e-07	Solute carrier family 39	Metabolism
SPATA18	2.19	2.16e-12	6.98e-09	Spermatogenesis associated 18	Metabolism
P3H2	2.10	2.28e-09	2.46e-06	Prolyl 3-Hydroxylase 2	Metabolism
F3	2.09	0.00037	0.027	Coagulation Factor III	Metabolism
GLS2	2.01	4.79e-14	3.1e10	Glutaminase 2	Metabolism
WDR63	2.84	7.16e-11	1.35e-07	WD Repeat Domain 63	Unknown
RN7SL519P	2.05	0.00061	0.037	Pseudogene	Unknown

Downregulated genes day2 versus baseline					
SERPINB10	-2.12	1.75e-06	0.00574	Serpin peptidase inhibitor, clade B member 10	Apoptosis
NR1D2	-2.57	9.63e-06	0.00201	Nuclear Receptor Subfamily 1, Group D, Member 2	Transcription
NR1D1	-2.29	0.00015	0.0142	Nuclear Receptor Subfamily 1, Group D, Member 1	Transcription
CIART	-2.38	9.14e-05	0.0103	Circadian associated repressor of transcription	Transcription
HIST1H1A	-2.56	8.31e-07	4.18e-06	Histone Cluster 1, H1a	Transcription
HIST1H1B	-2.04	6.70e-09	6.60e-06	Histone Cluster 1, H1b	Transcription
HIST1H3J	-2.00	8.31e-07	0.00033	Histone Cluster 1, H3j	Transcription
OXGR1	-2.04	8.93e-05	0.010	Oxoglutarate (Alpha-Ketoglutarate) Receptor	Cell signaling
PER3	-2.76	1.77e-05	0.0032	Period Circadian Clock	Metabolism
CYSLTR1	-2.91	8.72e-06	0.0019	Cysteinyl Leukotriene Receptor 1	Cell structure
KIF20A	-2.05	4.65e-08	3.22e-05	Kinesin Family Member 20A	Cell structure
PIK3C2G	-2.06	4.8e-06	0.00121	Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing gamma polypeptide	Cell growth

APPENDIX E: GENE LIST TSCC

GENES ALTERED IN MUCOSA					
Gene symbol	**FC**	**p-value**	**adj. p-value**	**Qualified Gene Ontology term**	**Function**
Baseline					
Mucosa - downregulated genes at baseline versus healthy controls					
LIFR	-2.73	2.09e-05	0.019	Leukemia Inhibitory Factor Receptor Alpha	Cellular differentiation, proliferation, survival
FKBP5	-2.48	0.00015	0.037	FK506 Binding Protein 5	Immune regulation, basic cellular processes
SPARCL1	-2.24	0.0002	0.041	SPARC Like 1	Cell adhesion, migration, and proliferation
MS4A4E	-2.30	9.06e-06	0.018	Membrane Spanning 4-Domains A4E	Cell surface signaling
PDGFRA	-2.11	1.74e-06	0.010	Platelet Derived Growth Factor Receptor Alpha	Cell surface tyrosine kinase receptor
Mucosa – up-regulated genes at baseline versus healthy controls					
RN7SL783P	2.54	0.00010	0.031	pseudogene	Unknown function
MTND5P8	2.17	0.0002	0.04	pseudogene	Unknown function

ABO	2.02	8.82e-07	0.001	Alpha 1-3-N-Acetylgalactosaminyltransferase	Enzyme, modifying surface glycoproteins
After seven days of radiotherapy					
Gene symbol	**FC**	**p-value**	**adj. p-value**	**Qualified Gene Ontology term**	**Function**
Mucosa - downregulated genes on day7 versus baseline					
HIST1H3B	-2.91	7.52e-08	0.000143	Histone Cluster 1, H3b	Transcription
HIST1H2BM	-2.75	1.6e-07	0.000251	Histone Cluster 1, H2bm	Transcription
CYSLTR1	-2.54	3.91e-05	0.0098	Cysteinyl Leukotriene Receptor 1	Cell structure
HIST1H3C	-2.39	9.08e-06	0.0039	Histone Cluster 1, H3c	Transcription
HIST1H3H	-2.17	4.53e-08	0.000105	Histone Cluster 1, H3h	Transcription
MOXD1	-2.16	6.19e-08	0.000128	Monooxygenase DBH Like 1	Metabolism
HIST1H1A	-2.12	0.00016	0.022	Histone Cluster 1, H1a	Transcription
HIST1H1B	-2.09	1.05e-08	3.14e-05	Histone Cluster 1, H1b	Transcription
MKI67	-2.00	2.58e-06	0.0016	Marker Of Proliferation Ki-67	Transcription
Mucosa – up-regulated genes on day7 versus baseline					
WDR63	2.67	1.09e-10	1.1e-06	WD Repeat Domain 63	Unknown

MDM2	2.29	6.77e-11	4.26e-11	MDM2 oncogene, E3 ubiquitin protein ligase	Apoptosis
EDA2R	2.26	8.38e-11	1.0e-06	Ectodysplasin A2 receptor	Apoptosis
POLH	2.17	3.22e-10	1.81e-06	Polymerase; DNA directed	Transcription
KRT16	2.15	0.00058	0.052	Keratin 16	Cell structure
Three weeks after RT cessation					
Gene symbol	**FC**	**p-value**	**adj. p-value**	**Qualified Gene Ontology term**	**Function**
Mucosa - downregulated genes after RT versus baseline					
ANKRD20A5P	-3.56	2.90e-07	0.0026	Ankyrin Repeat Domain 20 Family Member A5	Pseudogene
CYSLTR1	-3.11	3.92e-06	0.0082	Cysteinyl Leukotriene Receptor 1	Cell structure
SCIN	-2.50	9.09e-05	0.044	Scinderin	Cell structure
ANKRD20A11P	-2.47	4.93e-05	0.033	Ankyrin Repeat Domain 20 Family Member A11	Pseudogene
ANKRD20A9P	-2.32	1.2e-06	0.0052	Ankyrin Repeat Domain 20 Family Member A9	Pseudogene
CYP4F34P	-2.28	4.1e-05	0.032	Cytochrome P450 Family 4 Subfamily F Member 34	Pseudogene
TC2N	-2.13	6.47e-05	0.036	Tandem C2 Domains, Nuclear	Metabolism
IL1R2	-2.12	3.37e-07	0.0026	Interleukin 1 Receptor Type 2; cytokine receptor of the interleukin 1 receptor family	Immune response
Mucosa – up-regulated genes on day 21 versus baseline					

MIR31HG	5.30	5.71e-05	0.035	Non-coding microRNA no 3	Non-coding mi-RNA
CCAT1	3.08	1.08e-05	0.018	Colon Cancer Associated Transcript 1	Non-coding RNA
PTPRZ1	2.93	0.000103	0.047	Protein Tyrosine Phosphatase, Receptor Type Z1	Transcription
Mucosa - downregulated genes in patients with mucositis vs. no mucositis at baseline					
Gene symbol	**FC**	**p-value**	**adj. p-value**	**Qualified Gene Ontology term**	**Function**
LY6G6C	-3.78	2.53e-06	0.0995	Lymphocyte Antigen-6 G6C	Signal transduction Immune response
GENES ALTERED IN BLOOD CELLS					
Gene symbol	**FC**	**p-value**	**adj. p-value**	**Qualified Gene Ontology term**	**Function**
Blood - downregulated genes at baseline versus healthy controls					
RNU6-620P	-11.8	1.48e-12	5.80e-08	RNA, U6 small nuclear 620, pseudogene	pseudogene
RNU6-422P	-3.77	3.03e-08	0.00022	RNA, U6 small nuclear 422, pseudogene	pseudogene
RNU6-737P	-3.36	1.34e-07	0.00034	RNA, U6 small nuclear 737, pseudogene	pseudogene
RNU6-795P	-2.85	2.82e-06	0.0024	RNA, U6 small nuclear 795, pseudogene	pseudogene
RPS7P2	-2.63	2.14e-07	0.00044	Ribosomal protein S7 pseudogene 2	pseudogene
AGAP9	-2.61	6.15e-06	0.0039	ArfGAP With GTPase Domain, Ankyrin Repeat And PH Domain 9	GTPase-activating

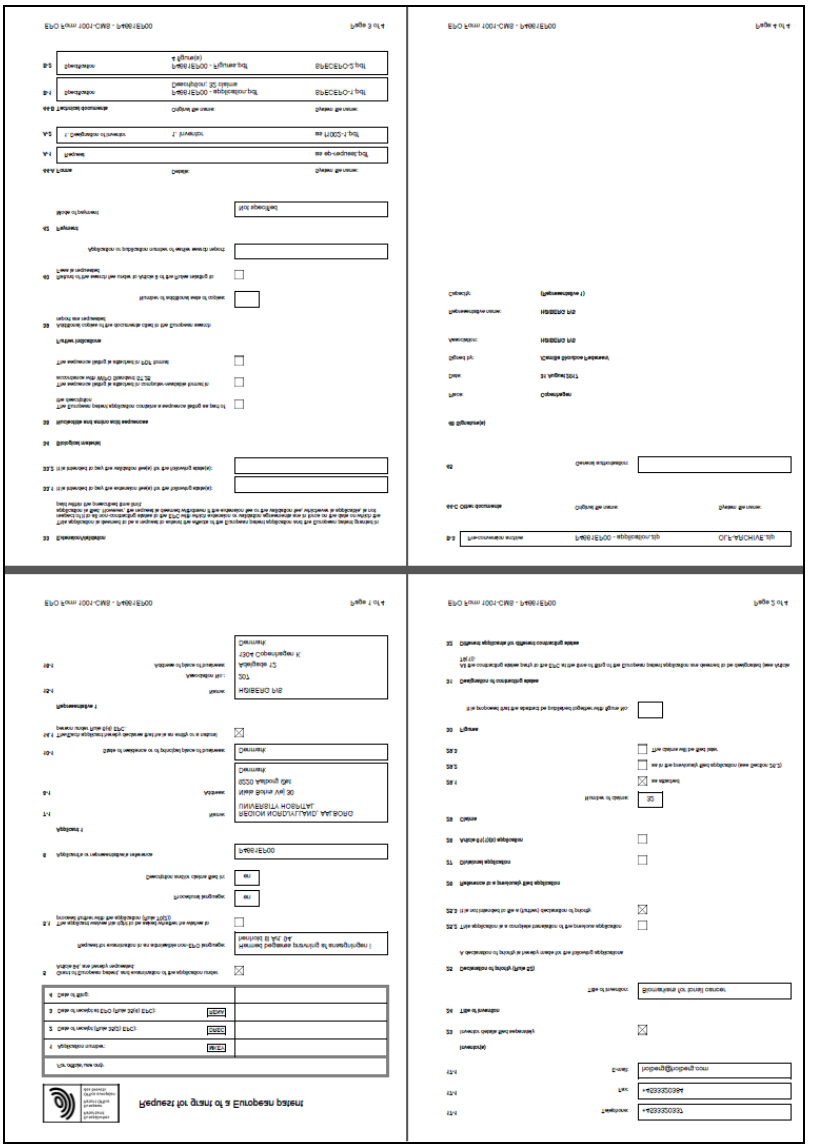
RNU6-336P	-2.45	5.72e-08	0.00025	RNA, U6 small nuclear 336, pseudogene	pseudogene
OAZ1	-2.26	6.81e-06	0.0040	Ornithine decarboxylase antienzyme 1	Cell growth and proliferation
RPL23AP64	-2.19	0.00012	0.018	Ribosomal protein L23a pseudogene 64	pseudogene
RNU6-1162P	-2.06	2.02e-05	0.0068	RNA, U6 small nuclear 1162, pseudogene	pseudogene
CCDC144B	-2.02	0.00074	0.043	Coiled-Coil Domain Containing 144B	pseudogene
RN7SL432P	-2.02	5.37e-07	0.00088	RNA, 7SL, cytoplasmic 432, pseudogene	pseudogene
Blood up-regulated genes at baseline versus healthy controls					
RNU6-622P	7.30	7.74e-09	8.62e-05	RNA, U6 Small Nuclear 622, Pseudogene	pseudogene
DUTP6	3.45	1.74e-06	0.0019	Deoxyuridine Triphosphatase Pseudogene 6	pseudogene
SSU72P8	3.44	1.07e-07	0.0014	RNA Polymerase II CTD Phosphatase Homolog, Pseudogene 8	pseudogene
RNU6-919P	3.37	1.06e-05	0.0051	RNA, U6 Small Nuclear 919, Pseudogene	pseudogene
RPS6P15	3.01	2.82e-06	0.0024	Ribosomal Protein S6 Pseudogene 15	pseudogene
RN7SL748P	2.44	1.59e-05	0.0061	RNA, 7SL, Cytoplasmic 748, Pseudogene	pseudogene
RPL10P4	2.33	2.88e-07	0.00051	Ribosomal Protein L10 Pseudogene 4	pseudogene
RPL21P133	2.32	6.39e-07	0.0010	Ribosomal Protein L21 Pseudogene 133	pseudogene

RN7SL290P	2.22	1.06e-05	0.0051	RNA, 7SL, Cytoplasmic 290, Pseudogene	pseudogene
OR5M4P	2.21	4.97e-05	0.011	Olfactory Receptor Family 5 Subfamily M Member 4 Pseudogene	pseudogene
RNU6-151P	2.19	1.58e-07	0.00036	RNA, U6 Small Nuclear 151, Pseudogene	pseudogene
RNU6-135P	2.19	1.29e-07	0.00034	RNA, U6 Small Nuclear 135, Pseudogene	pseudogene
RNA5SP116	2.18	0.00085	0.046	RNA, 5S Ribosomal Pseudogene 116	pseudogene
NUTM2D	2.13	0.00016	0.021	NUT family member 2D	unknown
RNA5SP54	2.06	8.49e-08	0.00030	RNA, 5S Ribosomal Pseudogene 54	pseudogene
RN7SL865P	2.05	0.00074	0.043	RNA, 7SL, Cytoplasmic 865, Pseudogene	pseudogene
RPS29P8	2.00	8.69e-07	0.0012	Ribosomal Protein S29 Pseudogene 8	pseudogene

APPENDIX F: GENE LIST AML

Table 3 Genes altered more than 1.5 fold (P < 0.05) in the buccal mucosa of patients with AML					
Gene symbol	FC	p-value	adj. p-value	Qualified GO term	Function
Baseline versus healthy controls					
RNU6-996P	2,04	2,28E-07	0,0067	RNA, U6 Small Nuclear 996, Pseudogene	unknown
LINC01975	1.66	2.33e-06	0.030	Long Intergenic Non-Protein Coding RNA 1975	unknown
Day 2 versus baseline					
HIST1H1A	-3.20	8.08e-10	3.18e-05	Histone Cluster 1, H1a	transcription
HIST1H2BM	-2.83	2.48e-06	0.024	Histone Cluster 1, H2BM	transcription
POLH	2.18	1.97e-06	0.024	Polymerase; DNA directed	transcription
NOTCH1	1.85	2.04e-06	0.024	NOTCH 1	cell signaling

Appendix G: Patent issued



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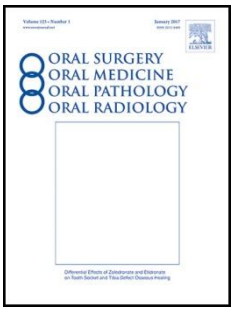

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A systematic review of molecular responses to cancer therapy in normal human mucosa

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Statement of Clinical Relevance: Clinical tools for pretreatment identification of patients likely to develop severe cancer therapy-related side-effects or measures to relieve these conditions are needed. We focus on identification of human phenotypes in the mucosa to guide further research in the field.

Abstract

Objective: Cancer therapy-induced inflammation of oral and gastrointestinal mucosa affects patients non-uniformly. Preventive strategies are limited; no biomarker exists for pretreatment identification of patients likely to be severely affected. Animal models are preferred for studying molecular responses in mucosa during chemotherapy, but translation into clinical practice is difficult. We performed a systematic review to retrieve papers that described molecular changes in human mucosa during cancer therapy.

Study Design: We searched MEDLINE and Ovid Embase searches for English-language literature from January 1990 to November 2016 and studies referenced in selected papers, that analyzed human mucosa from patients at risk of developing mucositis during cancer therapy. Two authors extracted data according to predefined data fields, including study quality indicators.

Results: We identified 17 human studies on chemotherapy (n=9) and radiotherapy (n=8), but no targeted therapy studies. Studies were heterogeneous regarding patient cohort, analysis methods, cancer treatment, biopsy timing, and correlations to clinical mucositis. Consequently, meta-analysis was not feasible.

Conclusions: Few human studies described the molecular responses of normal mucosa to cancer therapy. Studies were heterogeneous with sparse correlations to clinical mucositis. We proposed a model for acquiring data on treatment- and disease-specific phenotypes and transcriptomes for predictive or preventive initiatives.

Key words: Mucositis; stomatitis; gene expression; biomarker; chemotherapy; radiotherapy; targeted therapy; human

INTRODUCTION

Rationale

Mucositis is an acute, and potentially dose-limiting, adverse effect of cancer therapy. It presents as oral and/or gastrointestinal inflammation.[1,2] Upon chemotherapy (CT) initiation, mucositis appears clinically after 7-10 days, and it spontaneously resolves at approximately one week after treatment cessation.[3,4] For patients treated with radiotherapy (RT), a dose-response relationship was apparent[5,6]; mucositis generally appeared after a cumulative dose of 30 Grey.[7,8] Targeted therapy also induces mucositis, with mucosal alterations that clinically present as aphtous affections.[9-11]

At the molecular level, cancer therapy inflicts direct DNA damage.[12-14] Cytokines (TNF-α, IL-1β, and IL-6) enter the circulation and activate an inflammatory cascade (via NF-κB).[4,15-18] Both intrinsic and extrinsic apoptotic pathways are upregulated and mucosal integrity is compromised by inflammatory infiltrates and tight junction disruption.[19,20] This process leads to further tissue damage, involving matrix metalloproteinases (MMPs) and other effectors.[21] Additionally, cancer therapy disrupts the microflora, reducing its diversity and load; this imbalance paves the way for opportunistic infections or the reactivation of latent viruses.[21-30]

Nevertheless, patients are differentially affected by mucositis and the clinical impact has been thoroughly described previously.[3,9,31,32] Compared to mild occurrences (NM), ulcerative mucositis (UM) is associated with a high incidence of fever, diarrhea, nausea, and opportunistic

infections. Patients with UM often require severe pain relief, may require a feeding tube, and generally require prolonged hospitalization.[2,32-35] Consequently, UM continues to be a considerable burden to patients and the healthcare system.[37]

Objectives

The current model of mucositis pathogenesis is based primarily on animal studies.[4,18,21,38-41] It has been difficult to translate findings from those studies into clinical practice.[42] Therefore, current preventive options for mucositis are few,[37,43-48] and biomarkers are needed for the pretreatment identification of patients likely to develop severe mucositis.[49]

Consequently, the objective of this study was to provide an overview of the studies that analyzed the molecular changes in normal human mucosa during cancer therapy. The entire gastrointestinal tract has a common developmental history, and several studies have shown that different sections displayed a similar mucositis pathology.[4,39,50,51] Consequently, we conducted a systematic review to identify studies that described molecular changes in normal human mucosa (oral and gastrointestinal) from patients at risk of developing mucositis that recieved cancer treatment. Finally, based on our findings, we proposed a method to guide future research with a "presicion medicine" approach.

METHODS

This review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).[52] A protocol was registered at the International Prospective Register of Systematic Reviews (PROSPERO) database, accessible at https://www.crd.york.ac.uk/PROSPERO/display_record.asp?ID=CRD42017059447

Inclusion criteria

Only clinical studies that involved patients in cancer treatment that were at risk of developing mucositis were eligible for this review. For inclusion, the studies had to describe molecular changes in normal human oral mucosa and/or gastrointestinal mucosa

Exclusion criteria

Papers were excluded for the following reasons: (1) animal studies; (2) tissues other than mucosa were analyzed (e.g., blood or saliva); (3) only brush biopsies were studied; (4) only histomorphology was described; (5) published only as a conference abstract; (6) published in a review; and (7) published in a language other than English.

Information sources

We identified studies by searching the electronic databases, MEDLINE and Ovid Embase (January 1990 – November 2016). Moreover, we scanned the reference lists of the selected papers to identify additional articles. An updated search was performed at the end of December, 2016.

Search

We determined an appropriate search strategy by combining medical subject headings (MeSH) and EMTREE headings with natural language terms. Based on that strategy, we used the following search terms: mucositis, stomatitis, gene expression, biomarker, chemotherapy, radiotherapy, targeted therapy, cancer, neoplasms. A full detailed description of the search strategy appears in the Supplementary Table, S1 (available at [URL/link *]). We removed duplicate references with the EndNote tool.

Study selection

Two independent reviewers (MM, CS) screened records and selected papers according to inclusion criteria. Any disagreement was resolved by consensus. The abstracts of the selected papers were

screened based on the exclusion criteria. The flowchart of the literature retrieval process is shown in Figure 1.

A total of 5184 records (after removing duplications) were identified and screened according to the inclusion criteria. This process resulted in the removal of 5157 records. Of the remaining 22 records, the abstracts were screened, and an additional 4 records were removed, because only histomorphology or brush biopsies were studied.[53-56] Next, the full text of 18 papers were assessed, and four papers were removed; two papers were only available in the form of a conference communication, [57,58] one was published in German,[59] and one compared normal tissue to tumor tissue.[60] Finally, 14 full-text articles were deemed eligible, and these were included in the analysis.[17,18,50,61-71] The reference lists of the retrieved papers were screened for additional papers, and three more papers were identified.[72-74] The final analysis included 17 papers (Table I). Of the 17 papers, nine analyzed CT treated mucosal tissues, and eight described RT treated mucosal tissues.

Data collection process

Data was extracted from the papers by two independent reviewers (MM, CS), according to predefined criteria. We extracted the following data: first author's name, year of publication, number of study subjects, number of healthy control subjects, type of cancer, type of treatment, type of tissue analyzed, time from commencing therapy to tissue biopsy, method used to analyze tissue, performance of clinical mucositis assessment (yes/no) and the method used, biological process analyzed, and study conclusions.

Risk of bias assessment

The selected papers were evaluated for the risk of bias with the Meta Analysis of Statistics Assessment and Review Instrument (MAStARI) from the critical appraisal tools for Comparable Cohort / Case Control Studies.[75] The risk of bias was classified as high (up to 49% score "yes"),

moderate (50% to 69% score "yes"), or low (more than 70% score "yes"). A risk of bias summary is shown in supplementary Table S2 (available at [URL/link *]).

RESULTS

Chemotherapy

A biomarker for mucositis severity was proposed in only one of the nine studies on CT treated patients.[65] That study included a homogenous cohort of 50 patients with colorectal cancer that received 5-Fluorouracil (5-FU). They studied thymidylate synthase (TS), a key enzyme that controls DNA replication, which is targeted by 5-FU. They reported that low expression of TS was associated with grades 2-5 mucositis, based on the WHO mucositis assessment scale.

A global gene expression analysis (GGE) was applied in two studies.[62,63] One study examined archived autopsy specimens from nine patients with various cancers that received different types of CT (doxorubicin, cyclophosphamide, or 5-FU).[62] They found no genes that were correlated with clinical data, and the time from CT to biopsy was variable miscellaneous. However, that gene expression analysis showed a common trend in patients that developed mucositis. They found upregulations in genes involved in DNA repair, the response to DNA damage, innate immunity, inflammation, and bacterial invasion. Despite robust statistical analyses, that study was weakened by its lack of clinical data, lack of a healthy control group, and the heterogeneity in the patient cohort. Nevertheless, that study served as proof-of-concept that gene expression data may be successfully retrieved from archival material, despite RNA degradation.

The second GGE study described molecular changes in buccal mucosa from four patients treated with cytarabine/daunorubicin for acute myeloid leukemia (AML).[63] Clinical data on mucositis was collected, but it was not correlated to the microarray data. However, they reported that, among eight significantly altered genes, the gene that encodes argininosuccinate synthase 1

(*ASS1*) was three-fold downregulated; *ASS1* suppresses nitric oxide production, and consequently, promotes p53-mediated apoptosis. Moreover, they found that a gene encoding a zinc transporter (*SLC39A6*) was three-fold upregulated. The zinc transporter is involved in the epithelial-mesenchymal transition and tissue repair. Those results were validated in a polymerase chain reaction assay. Although strict statistical analyses (LIMMA and SAM packages with significance set at p-values <.01) were performed, only four patients were included; therefore, the results were considered preliminary.

TUNEL is a labeling method for detecting apoptotic cells in tissues. This technique was applied in two studies.[50,72] Both studies included cohorts (n=20 and n=23) of patients with various cancers (e.g., breast, gastric, colorectal, lung, non-Hodgkin´s lymphoma, and chronic myeloid leukemia), and the patients received various types of chemotherapy. A seven-fold increase in apoptotic activity was observed in the intestinal mucosa the first day following chemotherapy. Apoptosis gradually declined, and the tissue returned to normal after 16 days.[72] The same pattern was observed in oral mucosa, where apoptosis gradually increased, peaked on day three, then declined, and the tissue returned to normal after 11 days. However, in both studies, the apoptosis pattern was not correlated with the grade of mucositis.

The pro- and anti-apoptotic family of Bcl proteins was studied using the patient cohort studies by Keefe et al. 2000.[64,72] Among the Bcl proteins, p53 is a transcription factor involved in initiating apoptosis, cell cycle arrest, and DNA repair. They found that p53, Bax, and Bak levels increased within one day following chemotherapy, and Mcl-1 levels decreased. However, those results were not correlated to clinical mucositis.

The inflammatory mediators, cyclooxygenase2 (COX2), prostaglandin E synthase (mPGES), and nuclear factor-kappa-B (NF-κB) were studied in patients with various solid cancers that had received various types of CT.[31,17] A correlation was demonstrated between mucositis severity and

elevated levels of COX2 and mPGES observed at10 days after CT administration. However, this correlation was not statistically significant, probably because it was based on only three subjects.[17] NF-κB and COX2 were also elevated after CT, but these changes were not correlated to mucositis severity.[31]

Tissue permeability and inflammatory mediators in the oral mucosa of 23 patients from the Gibson et al 2006 study were analyzed with immunohistochemistry (IHC).[50,61] They found alterations of the following proteins in these tissues: claudin-1, ZO-1, occludin; IL-1β, IL-6, TNFα, MMP-2, and MMP-9. The results were not correlated to clinical mucositis.

Radiotherapy

The eight studies retrieved on mucosa affected by RT applied primarily IHC (no GGE analysis). They probed tissues for selected inflammatory, apoptotic, and proliferative markers.[66-71,73,74] Seven studies analyzed oral mucosa in patients with head and neck cancer,[66,67,69-71,73,74] and one study analyzed archived radiated colonic mucosa (Table II).[68] Most of these studies showed some degree of correlation between the molecular data and clinical mucositis. However, no specific biomarker was proposed in any of these studies.

One study identified a correlation between mucositis severity and both short- and long-term elevated expression of 27E10 in submucosal macrophages.[73] Other macrophage subtypes (markers 25F9, CD163), granulocytes (marker CD15), and T-lymphocytes (markers CD3, CD4, CD8) were not similarly altered in expression.

Inflammation was generally the dominant theme throughout these studies. A transmembrane glycoprotein highly expressed in macrophage lineages, CD68, was elevated in patients expressing the level of grade 1 mucositis.[67] Many leukocyte subtypes (e.g., CD106-expressing cells) were downregulated after RT; in contrast, cells that expressed CD11b/CD18 and CD49d were upregulated, and persisted in the tissues for long periods.[70,71] However, these findings were not

correlated to mucositis severity. Another study compared the expression of the inflammatory markers, NF-κB and COX-2, in patients that received either short-term radiotherapy or long-term RT combined with 5-FU. They found no differences in expression, which suggested that inflammation persisted in radiated tissues.[68]

Investigations of endothelial permeability markers (CD54,CD106, and E-selectin) showed that the pattern of expression of these markers changed with both short-term and long-term treatments. Moreover, the transmembrane membrane adhesion signaling protein, beta2-integrin, was elevated, but beta1-integrin remained unchanged with radiotherapy.[74]

Three studies demonstrated epithelial proliferation and raised levels of cytokeratin in response to RT.[66,67,69] Following an initial period of apoptosis, epithelial proliferation started during the first week after radiotherapy.[67,69] IHC results showed the presence of Ki-67, the proliferation marker, and increased expression of [^{3}H]-TdR, a marker of DNA synthesis. These findings were demonstrated by comparing expression in tissues with grade 1 mucositis to expression in tissues without mucositis from the same patients.

DISCUSSION

The mucosa is composed of a multi-layer of epithelial cells with a high turnover rate supported by connective tissue. Originally, cancer therapy-induced mucositis was considered to be solely the result of genotoxic injury (apoptosis). It was thought to primarily affect cells in the basal stem cell layer which are continuously proliferating, and therefore, are particularly chemo- or radiosensitive.[76,77]

Murine models have been preferred in studies of the molecular biology of mucositis. Breaking the mucosal barrier in a neutropenic or mucositis affected patient by taking a biopsy may potentially allow bacteria or fungi to enter the blood stream. This risk of sepsis may have restrained researchers

or ethic committees to approve these kind of studies.[17] The animal studies have demonstrated that mucositis pathology is driven by apoptosis and inflammation.

The mechanisms are initiated immediately upon cancer therapy induction, both in the submucosa and in the epithelium, before any clinically visible damage occurs.[4,18,19,38,78,79] However, the integrity of the mucosa arises through a variety of mechanisms, including antigen-presenting dendritic cells (DCs). As part of the innate immune surveillance system, DCs monitor changes in oral microflora. This surveillance involves sampling the antigenic environment, and then presenting a "peptide menu" to T-cells, via their cell surface major histocompatibility complex class II receptors (MHC Class II).[80] Thus, DCs are highly important in the induction of tolerance or inflammation.[81] Although DCs are also present in intestinal mucosa, the concentration of DCs is considerably higher in buccal mucosa than in mucosa in other regions.[81-84]

A comparison of human and mouse model responses to inflammation has shown poor correlations; they displayed different alterations in gene expression, particularly those involved in B-cell receptor signaling, macrophage and monocyte function, and the expression over time of the alpha chain of the class II histocompatibility antigen, human leukocyte antigen-D-related (HLA-DR).[85] Thus, it remains debated whether mouse models are relevant to studies of human disease. Due to the complexity of the factors involved in mucositis pathology (host response, microbiome, treatment modality, severity), the mechanisms underlying this condition might be best addressed in studies of human tissues.[42,86] Additionally, in animal models, oral mucositis often must be provoked, for example, by scratching the mucosa, which may distort the results.[79,87,88] Finally, when drugs were given to reduce the damage caused by RT by blocking some of the molecular pathways described in previous studies, they were not successful in reducing mucositis in clinical settings.[43,44,89,90]

The studies retrieved in this systematic review reassert that apoptosis is not the only factor involved in mucositis pathogenesis. Endothelial permeability is increased, inflammatory cells are attracted, and an array of inflammatory mediators is detectable in the tissues. Moreover, tight junction permeability is increased, which leads to mucosal microbiome involvement. Early defense mechanisms are instituted through anti-apoptotic mediators, restoration of epithelial proliferation, and cytokeratin production. Finally, short term microvasculature damage (aggregation of blood components and coagulation) may prolong the effect of CT locally because of reduced ability to remove damaging agents or long term damage in RT affected mucosa (telangiectasia, sclerosis and fibrosis) may impair healing . [68, 93,94]However, of the retrieved papers, only two studies found a correlation between the molecular events in the tissues and the severity of clinical mucositis.[65,73] Therefore, no meta-analysis was possible, due to the heterogeneity in study designs.

Several studies implied that a strong genetic component was associated with mucositis. Some genomic polymorphisms had predictive value, including modifications in genes that encoded drug-metabolizing enzymes, e.g. DRYD, the UGT1A1*28 allele or polymorphisms in genes that encoded p53 and MDM2.[93,94,95,96] A SNP-based Bayesian network was constructed, based on saliva-extracted DNA from 216 patients with various hematologic malignancies that were treated with human stem cell transplantation (HSCT). This SNP network could predict severe mucositis with a predictive validity of 81.2%.[53] That study assumed that only the type of drug, not the type of cancer, influenced the incidence of mucositis. In a second study, which included 972 patients suffering multiple myeloma and treated with HSCT, eleven SNPs located near the MMP13 gene were associated with mucositis grade 2-4.[54] But, to our knowledge, the findings from the above-mentioned studies have not been implemented clinically to adjust or individualize chemotherapy, with the aim of reducing the incidence of mucositis.[97] This fact is partly due to the low positive

predictive value of the tests.[98-100]. However, these data offer the potential for selecting patients for enrollment in clinical trials that aim to develop prevention strategies.[101]

Currently, there is a lack of studies that integrate phenotypic data with genomic data in the progress towards finding robust predictive biomarkers for mucositis. The importance of using human tissues to reveal phenotypes that may guide genotyping studies was well illustrated in a study by Santini et al.[65] Those authors found that low expression of thymidylate synthase, a key enzyme in the control of DNA replication and a target for 5-FU in tissues, was associated with grades 2-5 mucositis. A later study revealed that this result was due to polymorphisms in the gene that encoded thymidylate synthase.[102] Another recent study conducted under similar conditions (identical disease, treatment, and time interval for biopsy) proposed that differential gene splicing may account for heterogeneity in mucositis phenotypes.[103] They described a splice variant of HLA-DRB1 in epithelial dendritic cells that distinguished patients with UM from patients with NM.

We suggest conducting a number of pilot studies that are similar in nature to the studies described in this literature review. But, the pilot studies should have comparable designs and should be conducted under similar conditions. The design should include serial human mucosa biopsies with concurrent draws of peripheral blood, and patients with high-risk mucositis should receive different treatment regimens. Moreover, "omics" methodologies should be applied to the tissue samples to investigate correlations between molecular and clinical data.

This proposed model has some limitations. One limitation is the number of times that we can sequentially biopsy any one patient. However, this limitation may be even more restrictive in animal studies, because animals are typically sacrificed at biopsy.[38,104] Another limitation is the risk involved with taking mucosal biopsies during active cancer treatment. Breaking the mucosal barrier for a biopsy puts the neutropenic patient at risk of developing a complicating infection. This concern may complicate the ethics of these studies, which may impede institutional approval.[17]

However, among the human studies included in this review, no complications related to taking a biopsy were reported. Moreover, no biopsy-related complications were reported in a recent study on 10 patients with multiple myeloma that underwent 30 biopsies during HSCT treatment.[103] Strict sterility during the biopsy procedure and tight suturing may prevent infectious invasion; furthermore, the wound healing mechanisms were apparently intact during the cancer therapy.

The proposed model includes detailed, chronologic, molecular analyses of the human mucosa before, during, and after treatment, with clinical parameter measurements. The aim of this approach is to reveal the molecular pathways associated with mucositis phenotypes. International collaborations will be important for adding volume and structure to these studies. However, they must be conducted with standardized data sampling techniques, and overall, they must be aligned with the concept of precision medicine, as proposed by the National Research Council.[105,106] The ultimate goal would be to generate a drug- or disease-specific UM classification system, which could provide relevant, precise taxonomy of the pathologic processes. This information would support clinical decisions in determining the most appropriate care for each patient.

CONCLUSIONS

Few human studies have described the molecular responses of normal mucosa to cancer therapy. Those studies are heterogeneous in nature, with sparse correlations that point to markers for clinical mucositis. Nevertheless, some studies have revealed that disease- and treatment-specific transcriptomes exist. Therefore, we have proposed a model for future studies, which will facilitate the discovery of transcriptomes correlated to treatment- and disease-specific phenotypes. That information will provide a basis for predictive and preventive initiatives.

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Figure 1. Flow diagram showing the procedure for selecting studies for this systematic review.

Table I. Publications retrieved from our systematic literature search, chemotherapy

Comment [A1]: AUTHOR: Two different versions of Table 1 and 2 captions were provided and the one in the manuscript has been used. Please check and confirm that it is correct.

Paper	No	Cancer type	Treatment	Tissue	HCG	Time from therapy to biopsy	Method of analysis	Mucositis measuring	Molecular or genetic alterations	Conclusions
Wardill 2016	23[1]	Various	Chemotherapy miscellaneous	oral mucosa	7	Day 0 up to +11	IHC	Yes#	claudin-1, ZO-1 occludin IL-1β IL-6 TNF MMP-2 MMP-9	Increased tissue permeability through tight junction damage. Increase in inflammatory cytokines IL-1β, IL-6, TNF, MMP-2 and MMP-9. No correlation to clinical mucositis.
Mougeot 2013[33]	9	Various	Doxorubicin Cyclophosphamide 5-FU	oral mucosa archive	No	Miscellaneous, less than 30 days	GGE	No	Various	Proof-of-concept study showing extraction of eligible microarray data from FFPE-treated specimens. Alterations seen in pathways of apoptosis, DNA repair, innate immunity, inflammation and bacterial invasion.
Mougeot 2011[34]	3	AML	Cytarabine Daunorubicin	oral mucosa	3	Day 0; +2	GGE	No	ASS1 SLC39A6 CSNK1A1 DUT GBAS HNRPA0 MDM2 TRIAP1 TM7SF3	Pre-treatment AML-specific immune deregulation. Post-treatment inflammatory damage and p53 induced inhibition of apoptosis
Lalla 2010[35]	3	Various	HSCT miscellaneous	oral mucosa	No	Day -10; +10; +28; +100	PCR	Yes*	COX-2; mPGES	Inflammatory markers COX-2 and mPGES peaked at day +10. A correlation between pain scores and mucositis severity. Pilot study.
Logan 2007[36]	18	Various	Chemotherapy miscellaneous	oral mucosa	4	Day 0 up to +11	IHC	Yes#	COX-2; NF-kB	The inflammatory mediators COX-2 and NF-kB were not correlated to severity of clinical mucositis
Gibson 2006	20[1]	Various	Chemotherapy miscellaneous	oral mucosa	4	Day 0 up to +11	TUNEL	Yes#	terminal deoxynucleotidyl transferase (TdT)	Apoptosis occur before day 3 upon chemotherapy administration, starts to decline after 6 days, but has not returned to pre-treatment level at day 11.
Bowen 2005[38]	23[2]	Various	Chemotherapy miscellaneous	duodenal mucosa		Day 0; +1; +3; +5; +16.	IHC	No	p53 Caspase-3 Bax	Apoptotic markers of the Bcl-2 family, Bax/Bak and p53 increased upon initiation of chemotherapy and returned to normal level at

									Bak Mcl-1	day 3, whereas the anti-apoptotic member Mcl-1 decreased.
Santini 2004[40]	50	Colorectal	5-Fluorouracil	colonic mucosa	No	Day 0	IHC	Yes WHO	Thymidylate synthase	Low expression of thymidylate synthase, a key enzyme controlling DNA replication and a target for 5-FU was associated with grade 2-5 mucositis (WHO mucositis assessment scale).
Keefe 2000[39]	23[2]	Various	Chemotherapy miscellaneous	duodenal mucosa		Day 0; +1; +3; +5; +16	TUNEL	No	terminal deoxynucleotidyl transferase (TdT)	TdT a marker of apoptotic activity was seven-fold increased at day one.

Abbreviations: No=number of patients enrolled in the study. 1: Wardil 2016 and Gibson 2006 uses the same patient cohort. 2: Bowen 2005 and Keefe uses the same patient cohort. AML=acute myeloid leukemia. MM=multiple myeloma. HSCT=high dose chemotherapy with autologous stem cell support. 5-FU= 5-fluorouracil. HCG=Healthy Control Group. GGE=global gene expression; IHC=immunohistochemistry; PCR=Polymerase Chain Reaction. OMI=. COX=cyclooxygenase; mPGES,=microsomal prostaglandin E synthase. IL=interleukin. TNF=tumor necrosis factor. NK-kB=nuclear factor kappa-light-chain-enhancer of activated B cells. Bax, Bak, Mcl-1= members of the intrinsic apoptotic pathway Bcl-2 family regulated by p53 activated caspase-3. TUNEL= terminal deoxynucleotidyl transferase dUTP nick end labelling (a method for detecting DNA fragmentation). TdT= Terminal deoxynucleotidyl transferase. FFPE=formalin fixed and paraffin embedded. #Unspecific or no annotation of applied method; * Oral Mucositis Index (OMI) according to Schubert et al 1992. WHO=mucositis assessment according to World Health Organization (Quinn et al 2007)

Table II. Publications retrieved from our systematic literature search, radiotherapy

Paper	No	Cancer type	Treatment and dose	Tissue	HCG	Time from therapy to biopsy	Method of analysis	Mucositis measuring	Molecular or genetic alterations	Conclusions
Bonan 2007	10	HNC Mixed	Radiotherapy 46-60 Gy	oral mucosa	7	Before and after 2 weeks	IHC	Yes WHO	CD68 p53 Ki-67	Grade 1 mucositis is associated with increased number of inflammatory CD68 positive cells (macrophages), apoptosis (p53) and the presence of the cell proliferation marker Ki-67.
Bonan 2006	11	HNC Mixed	Radiotherapy 46-80 Gy	oral mucosa	No	Before and after 3 weeks	IHC	Yes WHO	Cytokeratin 1 Cytokeratin 6 Cytokeratin 10 Cytokeratin16	Grade 1 mucositis is associated with increased levels of cytokeratin 1, 6, 10, 14 and 16 compared to non-mucositis areas reflecting a defense towards radiation.
Yeoh 2005	28	Colorectal Mixed	Radiotherapy 25 Gy or 45 Gy/5-FU	colonic mucosa archive	No	Miscellaneous 4-65 days	IHC	Yes#	NF-kB; COX-2	Comparing short term RT with long term RT/5-FU the inflammatory markers NF-kB and COX-2 were equally expressed. Microvascular injury (telangiectasia, fibrosis and sclerosis) was associated with raised levels of NF-kB and COX-2
Dörr 2002	22	HNC	Radiotherapy 12-64Gy	oral mucosa	No	Miscellaneous 0-45 days	[^{3}H]-TdR in-vitro incubation	Yes*	[^{3}H]-TdR	Restoration of epithelial proliferation after initial apoptosis is initiated following the first week of radiation as measured by the expression of [^{3}H]-TdR, a marker of DNA synthesis.
Prott 2002 long term alter..	Ni	SCC HNC	Radiotherapy 60Gy	oral mucosa		Before, at 60Gy and 6-12 months after RT	IHC	No	ICAM-1 (CD54) VCAM-1 (CD106) E-selectin (CD62E) LFA-1 (CD11b/CD18) Mac-1 (CD11a /CD18) VLA-4 (CD49d) 27E10 25F9 RM3/1(CD163)	Subepithelial endothelium (markers CD54,CD106 and E-selectin) and subtypes of leucocytes (markers CD11b/CD18, CD11a /CD18, CD49d, 27E10, 25F9, CD163) were investigated. The expression of CD106 was downregulated after RT, whereas CD11b/CD18 and CD49d positive cells increased. After RT the radiated tissues have a reduced number of vessels and a different pattern of endothelial adhesion proteins as well as macrophages subpopulations.
Handschel 2001a late effect	Ni	SCC HNC	Radiotherapy 60Gy	oral mucosa	No	Before, at 60Gy, and 6 month after	IHC	No	ICAM-1 (CD54) VCAM-1 (CD106) E-selectin (CD62E) LFA-1 (CD11b/CD18) Mac-1 (CD11a /CD18) VLA-4	Sub-epithelial endothelium (markers CD54,CD106 and E-selectin) and subtypes of leucocyte (markers CD11a/CD18, CD11b/CD18, CD49d, 27E10, 25F9, CD163) were investigated. RT causes sub-epithelial migration of leucocytes which reverses to normal after cessation of RT. However some migrated cells persist in the tissues 6 month after RT (CD11b/CD18 and CD49d

									(CD49d) 27E10 25F9 RM3/1(CD163)	positive cells) A different pattern of adhesion molecules and macrophages subpopulations is are seen after RT.
Handschel 2001b increase	13	SCC HNC	Radiotherapy 60Gy	oral mucosa	No	Before, at 30Gy and 60Gy	IHC	Yes WHO	27E10 25F9 RM3/1(CD163) CD3 CD4 CD8 CD15	Subtypes of macrophages (markers: 27E10, 25F9, CD163), granulocytes (marker CD15) and T-lymphocytes (markers CD3, CD4, CD8) were investigated. Only 27E10 positive macrophages increased upon RT indicating an intermediate inflammatory response. There was a positive correlation between mucositis grade and the number of 27E10 positive macrophages.
Handschel 1999 irradiation	13	SCC HNC	Radiotherapy 60Gy	oral mucosa	No	Before, at 30Gy and 60Gy	IHC	Yes WHO	beta1-integrin (CD29) beta2-integrin (CD18) ICAM-1 (CD54) VCAM-1 (CD106) E-selectin(CD62E)	The transmembrane membrane signaling protein beta2-integrin increased while beta1-ingegrin stayed unchanged upon RT. The endothelial adhesion proteins CD54 and CD62E increased in expression while CD106 remained at low levels.

Abbreviations: No= number of patients enrolled in the study. Ni=not indicated; HNC=head and neck cancer. SCC= squamous cell carcinoma. Gy=Grey; 5-FU=5-fluorouracil; HCG=Healthy Control Group; RT=radiotherapy; IHC=immunohistochemistry; [^{3}H]-TdR= tritiated thymidine; WHO=mucositis assessment according to World Health Organization (Quinn et al 2007); #Unspecific or no annotation of applied method; *RTOG/EORTC scoring system according to the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer

Table S1 Algorithm for electronic search

Table S2 Risk of bias assessment

PLOS ONE

RESEARCH ARTICLE

Molecular Characteristics of High-Dose Melphalan Associated Oral Mucositis in Patients with Multiple Myeloma: A Gene Expression Study on Human Mucosa

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Abstract

Background

Toxicity of the oral and gastrointestinal mucosa induced by high-dose melphalan is a clinical challenge with no documented prophylactic interventions or predictive tests. The aim of this study was to describe molecular changes in human oral mucosa and to identify biomarkers correlated with the grade of clinical mucositis.

Methods and Findings

Ten patients with multiple myeloma (MM) were included. For each patient, we acquired three buccal biopsies, one before, one at 2 days, and one at 20 days after high-dose melphalan administration. We also acquired buccal biopsies from 10 healthy individuals that served as controls. We analyzed the biopsies for global gene expression and performed an immunohistochemical analysis to determine *HLA-DRB5* expression. We evaluated associations between clinical mucositis and gene expression profiles. Compared to gene expression levels before and 20 days after therapy, at two days after melphalan treatment, we found gene regulation in the p53 and TNF pathways (*MDM2*, *INPPD5*, *TIGAR*), which favored anti-apoptotic defense, and upregulation of immunoregulatory genes (*TREM2*, *LAMP3*) in mucosal dendritic cells. This upregulation was independent of clinical mucositis. *HLA-DRB1* and *HLA-DRB5* (surface receptors on dendritic cells) were expressed at low levels in all patients with MM, in the subgroup of patients with ulcerative mucositis (UM), and in controls; in contrast, the subgroup with low-grade mucositis (NM) displayed 5–6 fold increases in *HLA-DRB1* and *HLA-DRB5* expression in the first two biopsies, independent of melphalan treatment. Moreover, different splice variants of *HLA-DRB1* were expressed in the UM and NM subgroups.

Denmark Region [http://www.aalborguh.rn.dk/Forskning/Forskning-Nyhedsliste-Aalborg/Nyhed?id=c0b95ee0-4e0b-4fdd-bd92-5da0cb92a63e]; Herta Christensen Foundation [http://cvrapi.dk/virksomhed/dk/herta-christensens-fond/11681409]; Doctor Henrik Kopps Grant [http://www.laeger.dk/portal/page/portal/LAEGERDK/Laegerdk/Om%20L%C3%A6geforeningen/L%C3%A6geforeningen%20Nordjylland/Kontakt%20os/Speciall%C3%A6ge%20Heinrich%20Kopps%20Legat]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

Our results revealed that, among patients with MM, immunoregulatory genes and genes involved in defense against apoptosis were affected immediately after melphalan administration, independent of the presence of clinical mucositis. Furthermore, our results suggested that the expression levels of *HLA-DRB1* and *HLA-DRB5* may serve as potential predictive biomarkers for mucositis severity.

Introduction

For three decades, high-dose melphalan, supported with autologous stem cell transplantation (HSCT), has been a component of treatment for patients with newly diagnosed multiple myeloma (MM) [1]. However, melphalan induce adverse effects, including inflammation of the oral and gastrointestinal mucosa (mucositis) and prolonged neutropenia, which necessitates HSCT [2]. Melphalan induced mucositis occurs inconsistently, because although 80% of patients experience some degree of mucositis, only 40% are affected severely [2, 3]. Severe toxicity unfolds as a loss of mucosal integrity, severe diarrhea, and painful oral ulcers; i.e., ulcerative mucositis (UM) [3]. Complicated by bacterial or viral infections, these patients more often experience nausea, diarrhea, febrile episodes, and longer hospital stays compared to patients with mild or no mucositis (NM) [4, 5]. At present, international recommendations consist of infection control and palliative measures for pain relief [6]. Despite intense research efforts, no methods exist for preventing or reducing the duration of mucositis, and no predictive tests are available [7].

The mechanisms of action and metabolism of melphalan are well-described [8]. Melphalan alkylates DNA, which causes cross-links to form between DNA strands, and subsequently, DNA is degraded through apoptosis. The drug is administered intravenously, metabolized in the liver, and excreted through feces and urine. The degree of toxicity depends on renal function, body mass index (BMI), gender, and performance status [2, 9]. However, none of these factors are predictors of UM.

The current model of mucositis pathology is generalized across treatment regimens [10]. Initially, cancer therapy-induced DNA damage activates the intrinsic pro-apoptotic Bax/Bak and p53 pathways, and reactive oxygen species (ROS) are released [11, 12]. Simultaneously, damage to the extracellular matrix induces the release of inflammatory cytokines, which activate the extrinsic apoptotic pathway via tumor necrosis factor alpha (TNF-α) [13, 14]. This release is followed by an inflammatory response, which includes upregulation of the interleukins (IL) IL-1β, IL-6, IL-10, transforming growth factor-beta (TGF-β), nuclear factor-kappaB (NF-κB), and matrix metalloproteinases (MMPs) [15, 16]. This model is mainly based on murine studies and a few human studies, but to the best of our knowledge, no study has focused on patients with MM that were treated with melphalan.

Recent genome-wide association studies (GWAS) of patients that underwent HSCT have implied that UM development is associated with a genetic predisposition, primarily related to immune function [17, 18]. One study included 153 patients with miscellaneous malignancies that underwent HSCT, with the aim of building a predictive network for UM, based on 82 selected single nucleotide polymorphisms (SNPs) [17]. The network was subsequently tested in a cohort of 16 patients, and in the absence of any false positives, the predictive validity of the network was 81.2%. A later study included 972 patients with MM that underwent HSCT, and they identified 11 SNPs located near the matrix metalloproteinase gene that were associated

with UM and several known clinical risk factors. The sensitivity of predicting UM was 52% [18]. Apart from the low sensitivity, those studies were limited by their failure to identify phenotypes or causal relationships.

Here, we present a global gene expression study on oral mucosa biopsies and peripheral blood cell samples from consecutive patients with MM that were treated with high-dose melphalan and HSCT. This study aimed to identify new molecular factors that could predict severe oral mucositis.

Materials and Methods

Patients

This study included 30 patients, aged 18 years or older, recruited from the Aalborg University Hospital, from September 1st 2010 to September 1st 2012. Patients with MM (n = 20) were recruited from the Department of Hematology. Healthy individuals (CON, n = 10) were recruited for a control group from the Department of Maxillofacial Surgery. Of the 20 MM patients, seven withdrew consent before any intervention; one was missed due to earlier start of treatment, which was not communicated to the research unit; and two withdrew after the first biopsy without giving any reason. The remaining 10 patients provided three sequential buccal biopsies and peripheral blood samples. The first biopsy was obtained immediately before they received high-dose melphalan (day0); the second was obtained after the autologous stem cell reinfusion (day2); and the third was obtained during an outpatient control visit (day21). The CON group comprised 10 healthy, non-smoking, age- and gender-matched individuals. Controls provided one buccal biopsy and peripheral blood sample. One CON individual was later diagnosed with the autoimmune disease, psoriasis (CON09), and hence, this subject was not included in the statistical analysis. The North Denmark Region Committee on Health Research Ethics approved the clinical protocol (N-20100022). Informed written consent was obtained from all patients, in accordance with the Declaration of Helsinki.

All patients with MM underwent a comprehensive, initial evaluation, including a medical history and clinical examination. Age, gender, and Eastern Cooperative Oncology Group (ECOG) performance status were recorded at baseline, in addition to the subtype of MM and the time from diagnosis to entering HSCT. The criteria for determining the level of organ involvement at diagnosis was based on the degrees of elevated calcium, renal failure, anemia, and bone lesions (CRAB criteria) [19]. Furthermore, patients were screened for dental infections and, when indicated, these infections were eradicated prior to chemotherapy.

All patients with MM received a standard induction regimen, which consisted of cyclophosphamide 500 mg/m^2 delivered intravenously (i.v.) on days 1 and 8; bortezomib 1.3 mg/m^2 delivered subcutaneously (s.c.) on days 1, 4, 8, and 11; and dexamethasone 20 mg, delivered orally (p.o.) on days 1–2, 4–5, 8–9, and 11–12, repeated every third week, 3 times. After this treatment, patients were primed with cyclophosphamide 2 g/m^2 and recombinant granulocyte stimulating factor (rhG-CSF), before their circulating CD34$^+$ hematopoietic stem cells were harvested with leukapheresis [1]. Only patients without progressive disease were assigned to HSCT. These patients received a high dose of melphalan (200 mg/m^2), followed by infusion of autologous hematopoietic stem cells. All patients with MM had received standard antiviral, antifungal, and antibacterial treatment, according to department protocols.

Mucositis and diarrhea assessments

Signs of oral mucositis (OM) were recorded daily for patients with MM during the hospital stay (from administration of chemotherapy to discharge). OM signs were identified according to the WHO oral toxicity assessment worksheet [20], and they included subjective symptoms

(pain and ability to eat solid food) and objective findings (erythema, ulceration) in predefined regions of the mouth (lip, check, tongue, floor of the mouth, and soft palate). Grades 0 and 1 (NM) included increasing soreness, with or without erythema, but solid food could be taken. In grades 2 to 4 (UM), food intake gradually declined, due to pain and ulcerations, and parenteral feeding might have become necessary. The maximum OM grade recorded during treatment was considered the patient's general mucositis experience. Diarrhea was estimated according to the Common Terminology Criteria for Adverse Events (CTCAE), issued by The National Cancer Institute of the National Institutes of Health [21]. In grades 1 to 2, vomiting increases from one to two episodes in 24 h to three to five episodes in 24 h. Grade 3 included more than 6 episodes in 24 h, and grades 4 to 5 were considered life-threatening, and could gradually lead to death. Diarrhea data were gathered retrospectively, from medical records.

Biopsy

All biopsies were acquired in a standardized manner. First, the mouth was thoroughly rinsed with chlorhexidine and local anesthesia was applied (0.5 ml Citanest®: prilocain 30 mg/ml + felypressin 0.54 μg/ml; Dentsply, York, PA, US). Then, a 5-mm lens-formed biopsy of the buccal mucosa, approximately 1 cm inferior to the papilla parotidea, was taken with a scalpel. The wound was tightly sutured with resorbable vicryl 4.0 (Ethicon, Sommerville, NJ, US). Patients were instructed to rinse twice daily with chlorhexidine until suture removal, after 10 days. One-half of the biopsy was immediately immersed in RNA*later*™ (Ambion, Thermofischer Scientific, Waltham, MA, US) for 24 h; then, it was frozen at -80˚C until analysis. The other half of the biopsy was fixed in 10% neutral-buffered formalin, and within 1½ days, it was embedded in paraffin and maintained at room temperature until further preparation.

Peripheral blood

Within 2 h of taking the biopsy, 15 ml EDTA-mixed venous full blood was drawn. Mononuclear cells (MNCs) were isolated with an in-house standard purification protocol. This protocol follows the manufacturer's guidelines for Ficoll-Paque™ (GE Healthcare, Little Chalfont, Buckinghamshire, UK); density gradient centrifugation in Leukosep® tubes (Greiner Bio-One GmbH, Frickenhausen, Germany). Purified MNCs were suspended in freezing medium containing 10% dimethyl sulfoxide, in units of 5 million, vital frozen at -196˚C in liquid nitrogen, and stored frozen until analysis.

Gene expression

The frozen oral mucosa samples were homogenized with TRIzol® Reagent (Invitrogen, ThermoFischer Scientific), and total RNA was isolated with the mirVana™ miRNA Isolation Kit (Ambion/Invitrogen, ThermoFischer Scientific) according to the manufacturer's protocol. RNA amplification was performed with the Ambion® WT Expression Kit (Applied Biosystems, ThermoFischer Scientific), according to the manufacturer's instructions, starting with 100 ng total RNA, on a TP Basic Thermocycler, real time PCR instrument (Biometra, Göttingen, Germany). The quality of the RNA product was evaluated on the NanoDrop spectrophotometer and the 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, US). We prepared the RNA samples for hybridization to Affymetrix GeneChip Human Exon 1.0 ST Arrays with the Affymetrix GeneChip WT Terminal Labeling and Controls Kit (P/N 901524) (Affymetrix, Santa Clara, CA, US), according to the manufacturer's instructions. CEL files were generated with Affymetrix GeneChip Command Console Software and deposited at the NCBI Gene Expression Omnibus repository, under number GSE81979. A similar procedure was applied to analyze gene expression in MNCs isolated from blood samples.

Immunohistochemistry

We cut 4-μm-thick biopsy tissue sections and mounted them on glass coverslips. Following an in-house optimized protocol, tissues were stained with an antibody against the HLA class II Histocompatibility antigen, DR beta 5 chain (HLA-DRB5 center region) with a rabbit polyclonal antibody (no. OAAB06426, Aviva Systems Biology, CA, US). Normal tonsil tissue was used as a positive control. The stained slides were then scanned on a Hamamatsu NanoZoomer slide scanner and analyzed with NDP viewer software. To estimate the number of cells that stained positive for HLA-DRB5, each stained slide was searched for a hot spot; then, this spot was framed with a 0.75×0.4 mm (0.3 mm^2) rectangle; the area included approximately half lamina epithelialis and half lamina propria. We counted all cells in the frame that were distinctly stained with anti-HLA-DRB5 antibodies. The analyzer was blinded to the mucositis grade.

Statistical analysis

Power estimation of group size. To identify genes that varied more than two-fold between test points with a false discovery rate of less than 0.05% and a power of 90%, we applied the method described by Lee and Whitmore [22], implemented in the R-package, sizepower (Qui 2008). We found that 10 patients in each group were sufficient for detecting significant differences.

Statistical analysis. All statistical analyses were performed with R [23] version 3.2.0 and Bioconductor packages [24]. The p-values adjusted for false discovery rates were controlled with the Benjamini-Hochberg method [25], for each of the above tests. Adjusted p-values below 0.05 were considered significant.

The CEL files produced by the Affymetrix Expression Console and the probes were preprocessed and summarized to gene level with the RMA algorithm in the Bioconductor package 'affy', based on custom CDF files [26]. This preprocessing resulted in the gene expression levels of 38,830 genes for each Exon array each annotated with Ensembl gene (ENSG) identifiers. Patient CON09 was included in the normalizations of the gene expression data, but excluded in the statistical analysis.

With patient ID as a cluster variable, we used the linear model for microarray data (limma package in R), a mixed linear model, and an empirical Bayes approach to test for significant differences in gene expression levels between day2 and day0, and between day21 and day0 [27]. For the peripheral blood samples, we only compared day0 and day21 to baseline, because only two blood samples were analyzable for day2. We performed an unpaired test with the limma package to test for significant differences in gene expression between patients on day0 and controls.

The patients were divided into UM or NM groups, according to their mucositis experience. We used the limma package to detect significant genes that were differentially expressed between the two groups at each time point.

We applied the Mann-Whitney test to test for the relationship between mucositis severity and duration of neutropenia, leukopenia, and thrombocytopenia. We also used the Mann-Whitney test to evaluate differences between groups in the numbers of in-hospital days and years of progression free survival (PFS).

Results

The clinical characteristics and demographics of the included patients prior to HSCT are shown in **Table 1**. No signs of infection at the site of biopsy were reported. The clinical data collected during the HSCT and at follow up are shown in **Table 2**. UM (grades 2–4) was observed in 4 patients, and NM (grades 0–1) was observed in 6 patients. The average mucositis

Table 1. Patient characteristics and demographics upon enrollment in the study.

Patient	Age	Gender	ECOG	Weight	MM	CRAB	Induction cycles	Response induction	Standard HSCT	Diagnosis to HSCT
MM01	62	f	2	69	IgG-κ	B	3	No PD	Y	5.3
MM02	51	m	0	110	IgG-λ	B	3	No PD	Y	3.9
MM04	66	f	0	70	IgG-κ	R	3	No PD	Y	3.7
MM05	67	m	1	92	IgG-κ	B	3	No PD	Y	4.6
MM07	67	m	0	63	IgG-κ	C	3	No PD	Y	143
MM09	63	f	1	60	IgG-κ	B	3	No PD	Y	3.9
MM15	69	f	0	72	IgG-κ	A	3	No PD	Y	36.8
MM18	64	f	2	52	IgG-κ	R	3	No PD	Y	5.1
MM19	64	m	0	90	IgG-κ	B	3	No PD	Y	4.1
MM20	62	f	1	97	IgG-λ	B	3	No PD	Y	3.8

Abbreviations: ECOG = Eastern Cooperative Oncology Group performance status at baseline. MM = multiple myeloma subtype. CRAB = end-organ damage at diagnosis (C = hypercalcemia, R = renal failure, A = anemia, B = bone lesions) [19]. Induction cycles = Cyclophosphamide 500 mg/m^2 i.v. days 1 and 8; Bortezomib 1,3 mg/m^2 s.c. days 1,4,8, and 11; Dexamethasone 20 mg p.o. days 1–2, 4–5, 8–9, and 11–12, repeated every third week. PD = progressive disease, HSCT = high dose chemotherapy (melphalan 200 mg/m^2) with autologous stem cell transplantation. Diagnosis to HSCT = months between the diagnosis of MM and the HSCT procedure.

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scores were 1.5 (range 0–4) for the whole cohort, 3.3 for the UM group, and 0.3 for the NM group. The average diarrhea scores were 2.2 (range 1–4) for the whole cohort, 3.3 (range 3–4) for the UM group, and 1.5 (range 1–2) for the NM group. The average hospital stays were 22.6

Table 2. Patient clinical data during HSCT and at follow up.

Patients	Mucositis grade[1]	Diarrhea grade[2]	Neutro-penia[3] days	Leuko-penia[4]days	Thrombo-cytopenia[5] days	In-hospital days	PFS years	Status at follow up[6]
Patients with ulcerative mucositis								
MM04	2	3	4	4	10	41	4.5	CR
MM18	4	4	6	6	12	29	3.1	VGPR
MM19	4	3	10	8	10	21	3.0	VGPR
MM20	3	3	10	10	6	24	3.0	CR
Patients with no/mild mucositis								
MM01	0	2	10	10	14	24	4.1	Relapse
MM02	0	1	12	8	14	17	4.5	CR
MM05	1	2	6	6	14	21	3.2	Relapse
MM07	0	2	8	4	20	16	1.5	Relapse
MM09	0	1	10	8	10	17	1.0	Relapse
MM15	1	1	8	6	10	16	2.2	Relapse

Abbreviations: HSCT = high-dose chemotherapy (melphalan 200 mg/m^2) and autologous stem cell transplantation; PFS = progression free survival; the surrogate marker for overall survival was defined as the time from entering HSCT to disease progression, death, or follow-up [19]; CR = complete response; VGPR = very good partial response; Relapse = clinical relapse.

[1] Calculated according to WHO mucositis assessment scale [20]. Patients that experienced mucosal ulcerations during treatment were considered to have ulcerative mucositis, WHO grades 2–4; patients with only soreness or erythema were considered to have none/mild mucositis, WHO grades 0–1.

[2] Calculated according to the Common Terminology Criteria for Adverse Events [21]

[3] Neutropenia was defined as <0.5×10^6/l

[4] Leukopenia was defined as <0.5×10^9/l

[5] Thrombocytopenia was defined as <150×10^9/l.

[6]According to the International Uniform Response Criteria for multiple myeloma [19].

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days (range 16–41) for the whole cohort, 28.8 days (range 21–41) for the UM group, and 18.5 days (range 16–24) for the NM group. The difference in hospital stays was statistically significant ($p = 0.020$). The duration of neutropenia was not significantly different between UM and NM groups, but thrombocytopenia was significantly prolonged in the UM compared to the NM group ($p = 0.047$). PFS [28] was not statistically different between the UM and NM groups. The CON and MM groups were comparable in age (CON: age 58 y, range 47–78 vs. MM: age 63.5 y, range 51–69) and gender (CON: females 4/10 vs. MM: females: 6/10).

Analysis of gene expression in mucosa samples

All 40 biopsies (3×10 patients and 1×10 controls) provided gene expression profiles. No statistically significant differences in gene expression were found between the MM group on day0 and the CON group. Patients with MM showed no significant changes in gene expression between day0 and day21. However, 35 genes in patients with MM showed significantly different expression between day0 and day2 (**Table 3**). The gene expression levels were independent of clinical mucositis. The dominant gene alterations were observed in apoptosis-related genes, followed by genes related to inflammatory/immunologic response, transcription factors, and members of the Histone Cluster family. We also observed alterations in genes related to metabolism.

Gene expression related to mucositis grade

When we compared unsupervised gene expression profiles between NM and UM, we found that no genes were significantly differentially expressed in the blood. In contrast, in the biopsies, two genes of the major histocompatibility complex (MHC) Class II: *HLA-DRB1* and *HLA*-DRB5 were significantly differentially expressed at the first two time points (**Table 4**). Patients with UM and CON expressed the same low level of *HLA-DRB1* and *HLA-DRB5*, but patients with NM expressed significantly higher levels (**Fig 1**). The expression levels of *HLA-DRB1* and *HLA-DRB5* were independent of melphalan administration. Of the 10 CON subjects, one patient, CON09, showed high levels of *HLA-DRB1* and *HLA-DRB5* expression, similar to the levels observed in the NM group. We reopened the protocol and returned to the patient to reaffirm his health status. We found that subject CON09 had a mild case of psoriasis that was not reported at the baseline interview. An alternative splicing analysis revealed that patients with NM and the CON09 subject expressed a different isoform of *HLA-DRB1* (NM_001243965) than that expressed by patients with UM (NM_002124.1). However, the difference in *HLA-DRB5* expression between groups was not due to different isoforms.

Immunohistochemistry

In hematoxylin and eosin-stained specimens, no gross anatomical changes were observed in the epithelial or mesenchymal layers. In general, both the epithelium and stroma were represented in equal amounts. However, two specimens that were cut at a tangential angle that revealed only superficial layers were excluded from the histological analysis (MM15_1 and MM18_2). Generally, when present, cells that stained positive for *HLA-DRB5* were localized in the lower part of the epithelial layer, near the basal membrane, around the papillae, and in the upper part of the lamina propria. Faint, diffuse *HLA-DRB5* staining of the endothelium was not included in the assessment. Examples of high *HLA-DRB5* expression/low-grade mucositis (MM01) and low *HLA-DRB5* expression/severe-grade mucositis (MM18) are shown in **Fig 2**. A dotplot of the numbers of cells that stained positively for *HLA-DRB5* is shown in **Fig 3**, and these findings supported the gene expression analysis.

Gene expression in peripheral blood

Of the blood samples drawn from 10 patients with MM, we successfully performed gene expression profiles in 8 out of 10 drawn on day0 and day21, but only 2 out of 10 drawn on

Table 3. Genes altered in the buccal mucosa of patients with multiple myeloma.

Gene symbol	FC	p-value	adjusted p-value	Qualified GO term	Function
Upregulated genes day2 versus baseline					
MDM2	2.69	2.37e-15	3.07e-11	MDM2 oncogene, E3 ubiquitin protein ligase	Apoptosis
EDA2R	2.63	1.85e-15	3.07e-11	Ectodysplasin A2 receptor	Apoptosis
CUL9	2.25	1.26e-15	3.07e-11	Cullin-9	Apoptosis
INPPD5	2.18	8.39e-14	4.66e-10	Inositol Polyphosphate-5-Phosphatase	Apoptosis
TIGAR	2.17	7.08e-10	8.87e-07	Chromosome 1 open reading frame 5	Apoptosis
E2F7	2.06	2.60e-13	1.05e-09	E2F transcription factor 7	Apoptosis
NCR3LG1	2.70	1.14e-10	1.94e-07	Natural killer cell cytotoxicity receptor 3 ligand 1	Immune response
LAMP3	2.26	4.39e-06	0.0011	lysosomal-associated membrane protein 3	Immune response
TREM2	2.12	2.78e-09	2.92e-11	Triggering receptor expressed on myeloid cells	Immune response
FKBP5	2.04	6.66e-05	0.0082	FK506 Binding Protein 5	Immune response
POLH	2.42	1.60e-14	1.24e-10	Polymerase; DNA directed	Transcription
ARNTL	2.40	2.65e-06	0.00080	Aryl hydrocarbon receptor, nuclear translocator-like	Transcription
NFIL3	2.20	4.29e-05	0.0011	Nuclear factor, interleukin 3 regulated	Transcription
ABCA12	4.73	8.87e-07	0.00034	ATP-binding cassette sub-family A, member 12	Metabolism
CEL	4.64	3.68e-15	3,58e-11	Carboxyl ester lipase	Metabolism
CA2	2.57	9.99e-10	1.18e-06	Carbonic anhydrase II	Metabolism
SLC39A6	2.53	1.16e-10	2.00e-07	Solute carrier family 39	Metabolism
SPATA18	2.19	2.16e-12	6.98e-09	Spermatogenesis associated 18	Metabolism
P3H2	2.10	2.28e-09	2.46e-06	Prolyl 3-Hydroxylase 2	Metabolism
F3	2.09	0.00037	0.027	Coagulation Factor III	Metabolism
GLS2	2.01	4.79e-14	3.1e10	Glutaminase 2	Metabolism
WDR63	2.84	7.16e-11	1.35e-07	WD Repeat Domain 63	Unknown
RN7SL519P	2.05	0.00061	0.037	Pseudogene	Unknown
Downregulated genes day2 versus baseline					
SERPINB10	-2.12	1.75e-06	0.00574	Serpin peptidase inhibitor, clade B member 10	Apoptosis
NR1D2	-2.57	9.63e-06	0.00201	Nuclear Receptor Subfamily 1, Group D, Member 2	Transcription
NR1D1	-2.29	0.00015	0.0142	Nuclear Receptor Subfamily 1, Group D, Member 1	Transcription
CIART	-2.38	9.14e-05	0.0103	Circadian associated repressor of transcription	Transcription
HIST1H1A	-2.56	8.31e-07	4.18e-06	Histone Cluster 1, H1a	Transcription
HIST1H1B	-2.04	6.70e-09	6.60e-06	Histone Cluster 1, H1b	Transcription
HIST1H3J	-2.00	8.31e-07	0.00033	Histone Cluster 1, H3j	Transcription
OXGR1	-2.04	8.93e-05	0.010	Oxoglutarate (Alpha-Ketoglutarate) Receptor	Cell signaling
PER3	-2.76	1.77e-05	0.0032	Period Circadian Clock	Metabolism
CYSLTR1	-2.91	8.72e-06	0.0019	Cysteinyl Leukotriene Receptor 1	Cell structure
KIF20A	-2.05	4.65e-08	3.22e-05	Kinesin Family Member 20A	Cell structure
PIK3C2G	-2.06	4.8e-06	0.00121	Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing gamma polypeptide	Cell growth

Abbreviations: FC = fold change; GO = gene ontology annotation

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Table 4. Genes altered in the buccal mucosa of patients with multiple myeloma that displayed mild/no mucositis (NM) compared to those that displayed ulcerative mucositis (UM).

Gene symbol	FC	p-value	adjusted p-value	Qualified GO term	Function
NM versus UM day0, before melphalan					
HLA-DRB1	6.27	2.96e-07	0.00573	Human Leukocyte / Major Histocompatibility Antigen Class II DRB1 beta chain	Immune response
HLA-DRB5	5.64	2.7e-07	0.00573	Human Leukocyte / Major Histocompatibility Antigen Class II DRB5 beta chain	Immune response
NM versus UM day2 after melphalan					
HLA-DRB1	5.81	2.01e08	0.00039	Human Leukocyte / Major Histocompatibility Antigen Class II DRB1 beta chain	Immune response
HLA-DRB5	5.56	1.98e09	7.69e05	Human Leukocyte / Major Histocompatibility Antigen Class II DRB5 beta chain	Immune response

FC = fold change; GO = gene ontology annotation.

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day2 (MM20 and MM05). Consequently, we performed an unsupervised global gene analysis of peripheral MNCs by comparing the MM day0 samples to CON samples (disease vs. healthy) and MM day0 samples versus MM day21 samples (before vs. after treatment). We found that two genes that encoded B-cell surface markers (CD22, CD200) were downregulated in MM day0 samples compared to CON samples, and these genes were further downregulated on day21, though the differences from day0 were not significant. The levels of CD22 and CD200 expression were independent of the mucositis grade.

Discussion

This study described the gene signature of buccal mucosa samples from patients with MM during HSCT. We found that this signature was dominated by altered expression of inflammatory and anti-apoptotic genes, but expression was independent of the presence of clinical mucositis.

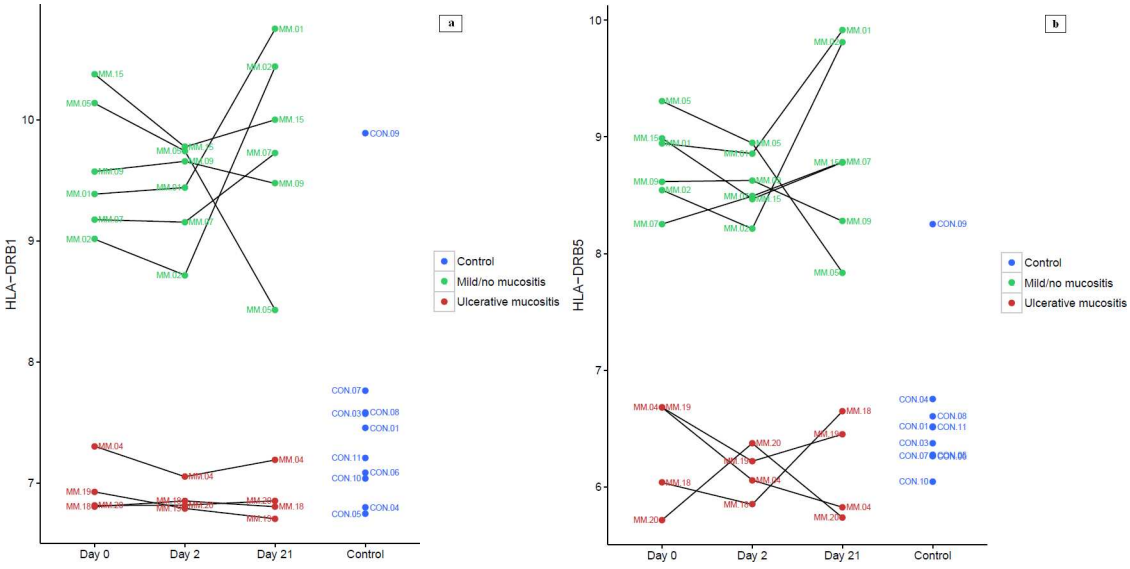

Fig 1. Genes differentially expressed according to mucositis grade. Expression of (a) *HLA-DRB1* and (b) *HLA-DRB5* genes in buccal mucosa biopsies taken at baseline (day0), two days (day2), and 21 days (day21) after high-dose melphalan therapy. Patients with mild/no mucositis (NM) express 6–8 fold more *HLA-DRB1* and 4–5 fold more *HLA-DRB5* than patients with ulcerative mucositis (UM). Melphalan treatment did not affect expression of *HLA-DRB1* or *HLA-DRB5* in either group. One healthy control (CON09) expressed the same high levels of *HLA-DRB1* and *HLA-DRB5* as those observed in the NM group. Subject CON09 was diagnosed with the autoimmune disease, psoriasis. Previous studies have reported that patients with psoriasis were 77% less likely to develop mucositis than patients without psoriasis [54].

doi:10.1371/journal.pone.0169286.g001

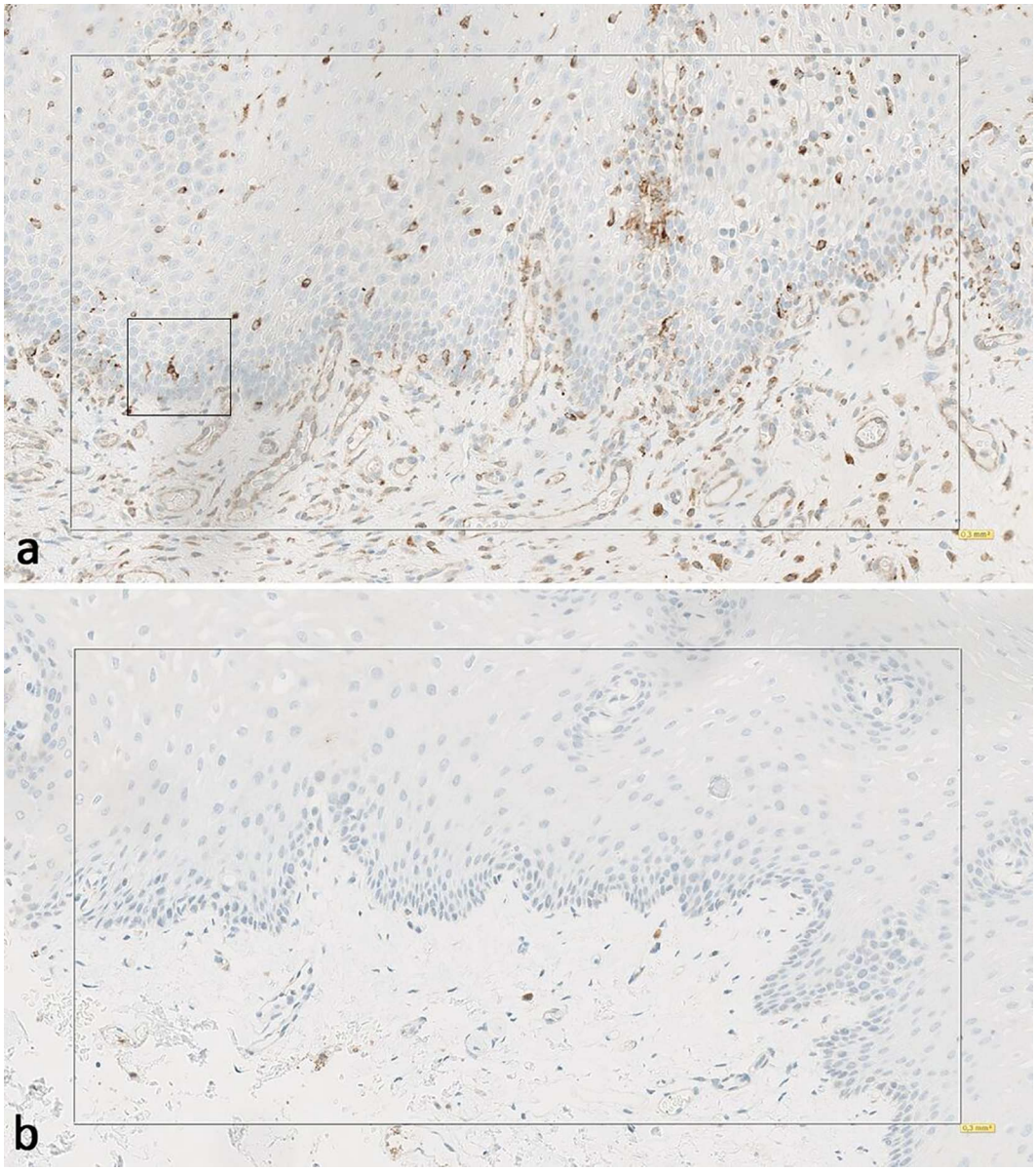

Fig 2. Immunohistochemical analysis of *HLA-DRB5* expression in oral mucosa biopsies. Oral buccal mucosa (×20 magnification) staining shows *HLA-DRB5* expression in the center region (a) High *HLA-DRB5* expression is observed in the patient MM01 with mild mucositis. (b) Low *HLA-DRB5* expression is observed in the patient MM18 with severe mucositis. Generally, when present, cells that stained positive for *HLA-DRB5* are primarily localized in the lower part of the epithelium, near the basal membrane, around the papillae, and in the upper part of the submucosa in close proximity to the basal membrane. A weak, diffuse *HLA-DRB5* staining of the endothelium is also visible. Normal tonsil tissue was included on the slide as a control; these appear identical in (a) and (b). The square insets highlight the morphology of one of the *HLA-DRB5* expressing cells that displayed extensions, similar to those observed in dendritic cells.

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Furthermore, we identified a specific isoform of the immunomodulatory gene, *HLA-DRB1*, which may serve as a biomarker for mucositis severity.

The process that leads to mucositis is triggered immediately upon initiation of cancer therapy and before any visible macroscopic damage [10]. Moreover, eventual UM coincides with neutropenia, within 7–10 days of starting chemotherapy. Therefore, to avoid compromising

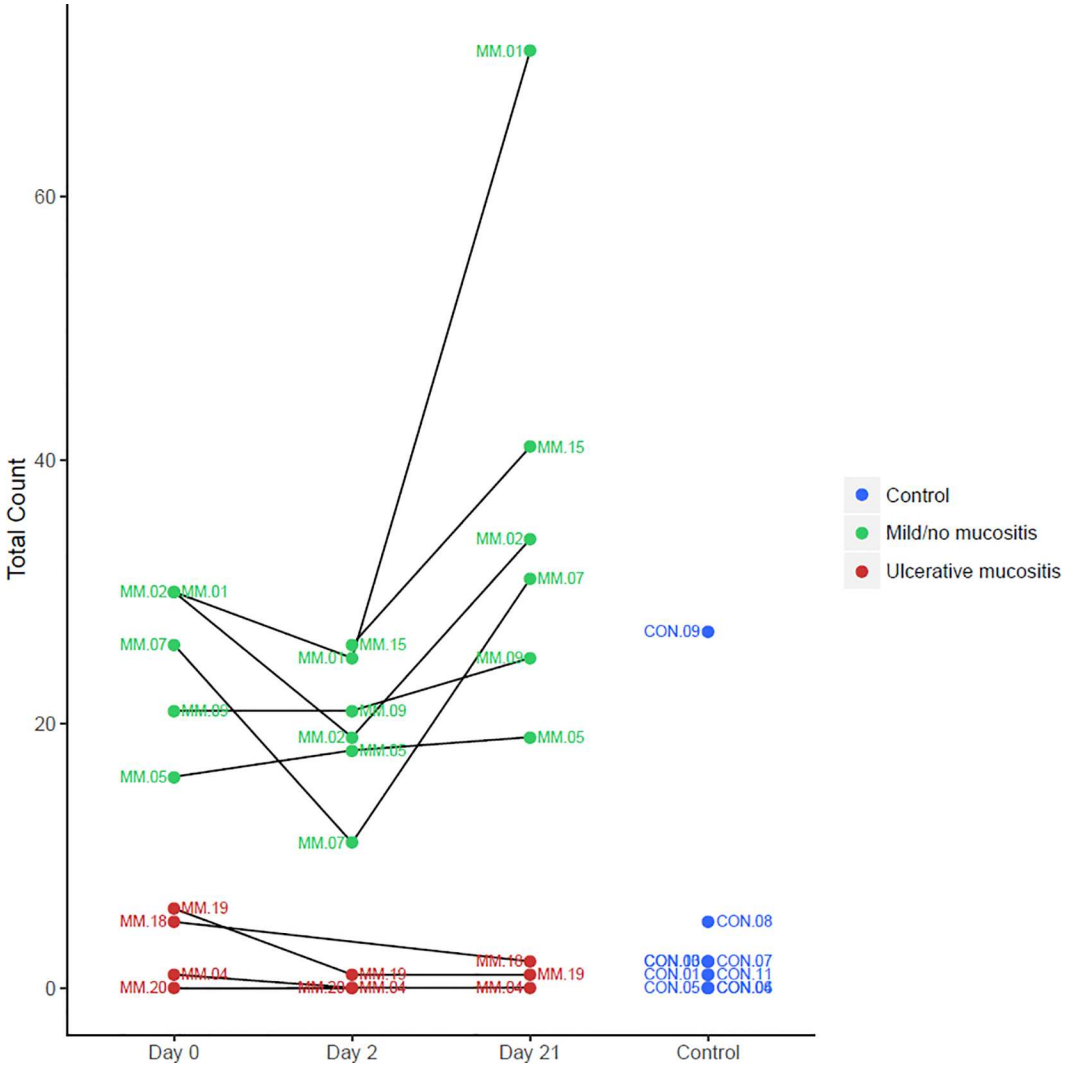

Fig 3. The number of cells that stained positive for *HLA-DRB5* in oral mucosa biopsies. A dotplot shows the cell count performed on oral mucosa biopsies that had been immunostained to detect *HLA-DRB5*. Each biopsy was examined to determine hot spots of expression; then, this section was framed with a rectangle of 0.75×0.4 mm (0.3 mm^2), and all distinctly stained cells within the rectangle were counted.

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patients with neutropenia, we acquired the second biopsy before the onset of neutropenia. With this approach, the biopsy was unlikely to comprise disintegrated tissue that, presumably, would be dominated by inflammatory mediators. Instead, we aimed to gain insight on the cellular processes that gave rise to the inflammatory state. Mucositis lasts for approximately 7 days, and then, it spontaneously resolves. We acquired the third biopsy on day 21, when the mucosa was fully restored.

Little is known about the effects of melphalan on normal epithelium, but in cancer cells, melphalan induces oxidative stress and upregulates a wide range of apoptosis-related genes [8, 29], consistent with our findings of *EDA2R* upregulation. *EDA2R* encodes a TNF receptor that mediates the activation of NF-κB and jun-N-terminal (JNK) pathways, which lead to caspase-initiated apoptosis. Previous studies reported that these pathways were activated in buccal mucosa of patients with various cancers that received HSCT [30–32], and in gastrointestinal mucosa of patients treated with 5-fluoruracil [33]. In contrast, we found upregulated expression

of *INPP5D*, which encodes a membrane protein in hematopoietic cells. The INPP5D protein negatively regulates JNK signaling and limits Fas-FasL-induced apoptosis in T-cells found at mucosal surfaces [34]. In addition, we identified five genes involved in suppressing the pivotal p53 apoptotic pathway. Four of these were upregulated: *MDM2*, *CUL9*, *E2F7*, and *TIGAR*; and one was downregulated: *SERPINB10*. The *MDM2* gene encodes a protein ligase that ubiquitinates p53, and thus, inhibits p53-mediated cell cycle arrest and apoptosis. A previous gene expression study on three patients with acute myeloid leukemia used the same time intervals between biopsies that we used, and they found *MDM2* upregulation [35]. In an array of studies, p53 has been identified as a key regulator of apoptosis, which leads to mucositis [11, 12, 36]. However, the gene alterations associated with apoptosis observed in our study, including *EDA2R*, did not depend on the level of clinical mucositis.

We found several genes related to transcription that were altered to favor DNA repair. For example, *POLH* was upregulated; *POLH* encodes a specialized DNA polymerase that accurately replicates UV-damaged DNA. Conversely, members of the histone cluster family (e.g., *HIST1H1A*), *NR1D1*, and *NR1D2* were downregulated. These results implied that defense against apoptosis and DNA damage was a central objective in the initial stage of mucositis. Importantly, this objective was independent of the mucositis grade, which implies that other factors must be involved in distinguishing UM and NM.

The immune response was activated at an early stage, through the upregulation of *TREM2* and *LAMP3* on day2. Both these genes encode DC membrane proteins that contribute to T-cell activation and mucosal inflammation. The *LAMP3* gene is specifically expressed in mature DCs [37, 38]. TREM2, which is expressed on both DCs and macrophages, can bind and phagocytose yeasts, Gram positive bacteria, and Gram negative bacteria [39], which are commonly present in the oral cavity [40]. Generally, DCs are potent antigen-presenting cells that respond to microbial exposure by secreting abundant cytokines; e.g., IL-12 and type I interferon. In turn, IL-12 mobilizes natural killer (NK) cells. The *NCR3LG1* gene, which encodes a ligand that triggers NK cells, was also upregulated [41]. Several studies have shown that an important aspect of mucositis pathology is the thinning of the epithelium, in combination with changes in the composition and concentration of the oral and gastrointestinal microbiota [13, 42, 43]. Our results confirmed the notion that the host immune response towards the microbiome played a dominant role, early in mucositis pathogenesis; however, these responses were not associated with mucositis severity.

Among several genes associated with metabolism, *ABCA12* and *CEL* were upregulated 4.7-fold and 4.6-fold, respectively, on day2. *ABCA12* encodes a membrane transporter protein primarily involved in the keratinocyte lipid-barrier that maintains homeostasis in the epidermis [44]. To the best of our knowledge, no previous study has described a role for *ABCA12* in the mucosa, but it most likely performs a similar function of barrier protection. *CEL* encodes a lipase with multiple functions in lipid metabolism; it is also expressed in macrophages [45]. The expression levels of both these genes were unaffected by clinical mucositis.

Gene alterations associated with clinical mucositis grade

When we compared the gene expression profiles between the six patients with NM and the four patients with UM, we found two genes that were more highly expressed in NM patients: *HLA-DRB1* and *HLA-DRB5* (**Fig 1**). The *HLA-DRB1* and *HLA-DRB5* genes are related members of the MHC Class II family, located on chromosome 6p21.32. They encode surface proteins that are almost exclusively expressed on specialized antigen presenting cells, including macrophages, B-cells, and DCs or Langerhans cells [46, 47]. These surface receptors function as a ligand for the T-cell receptor, and their primary function is to capture potentially foreign antigens on the

cell surface and to present them for recognition by CD4+ T-cells [48]. Thus, they form a communication between the innate and adaptive immune systems, and determine whether to bring forth resistance or tolerance, in addition to taking up and processing dying cells [49].

Several pharmacogenomic GWAS studies were recently performed on drug toxicity, which showed that HLA Class I and II paralogs were associated with toxicity [50] or inflammatory mucosal conditions [51, 52]. Even more interestingly, certain HLA-DRB1 alleles (HLA-DRB1*15) have been detected in patients with MM that were exposed to bisphosphonates and developed osteonecrosis of the jaw [53].

We conducted a search for alternatively spliced variants of *HLA-DRB1* and *HLA-DRB5* and found two isoforms of *HLA-DRB1* (NM_001243965 and NM_002124). According to the UCSC genome browser, NM_001243965 harbors six exons, and NM_002124 harbors an extended isoform within seven exons. We found that patients with NM expressed the longer transcript variant of *HLA-DRB1* (NM_002124), and patients with UM and healthy subjects (CON) expressed the shorter variant (NM_001243965). No splice variant was found for *HLA-DRB5*; however, that gene was expressed at different levels. We found that *HLA-DRB5* was expressed 4.5 to 5 times more frequently in the NM group than in the UM group, in the first two of three biopsies. These findings were confirmed in an immunohistochemical analysis of HLA-DRB5 in the biopsies (**Fig 3**). Furthermore, cells that stained positively for HLA-DRB5 were localized primarily in the epithelium and submucosa, relatively close to the basal membrane, and these cells displayed a morphology similar to DCs (Langerhans cells).

In contrast to the other healthy subjects, CON09 (a patient with psoriasis) expressed the long transcript variant of *HLA-DRB1* and a high level of HLA-DRB5 protein, similar to patients in the NM group. Previous reports have indicated that patients with psoriasis are 77% less likely to develop mucositis [10, 54]. Psoriasis is an auto-inflammatory skin disorder with reduced apoptosis. It is known that patients with psoriasis express certain *HLA-DRB1* alleles [55]. Our results suggested that mechanisms related to inflammatory and/or apoptotic pathways may be common in psoriasis and low-grade mucositis in MM. In addition, *ABCA12* expression was upregulated in CON09 compared to the other healthy subjects. Previous gene expression studies on patients with psoriasis confirmed this finding [56].

The two recent GWAS studies on patients that received HSCT identified SNPs near the locus of MMP and other genes related to inflammation, but none related to *HLA-DR* [17, 18]. Other recent studies found a major role for MMPs in mucositis pathology [57]. We did not find any changes of that nature in our material. The phenotypes described in our study may provide additional information to guide future GWAS studies [50]. Recent studies have shown that induction therapy with immune modulating agents reduced the frequency and severity of mucositis [58]; our results may provide additional knowledge to elucidate the development of those therapies.

In peripheral blood, we did not find any differences in gene expression between NM and UM groups, at any time point. However, among all patients with MM, *CD22* and *CD200* were downregulated on day0 compared to controls. Both these genes encode cell membrane glycoproteins of the immunoglobulin superfamily. *CD200* is expressed in multiple cell types, including B-cells, T-cell subsets, DCs and endothelial cells. In contrast, *CD22* is exclusively expressed on mature B-cell lineages [59, 60]. Low CD200 expression has been linked to prolonged survival among patients with MM [61]. In our cohort, the lowest CD200 expression levels were observed among patients with UM; however, the levels were not significantly different between UM and NM groups.

Study strengths and limitations

There was some concern that breaking the mucosal barrier by taking a biopsy during chemotherapy might lead to potential fatal infections. We could reject this concern, because none of

our patients experienced any infection related to the biopsy; only mild discomfort was reported. This finding was also reported in previous studies [15, 35, 36, 62]. Therefore, we concluded that our method would be safe for patients undergoing HSCT, provided that the second biopsy is taken before the onset of neutropenia. The major limitation of the study was the low number of subjects. We designed the study to identify genes that were altered by more than 2-fold between time points, with a false discovery rate of less than 0.05% and a power of 90%. However, because our method of harvesting human mucosa during high-dose melphalan treatment was controversial, we sought to include the least possible number of patients required to draw valid conclusions. However, we recognize that the power of this study was set to estimate any fold-changes above two, and false negative findings may be concealed. Consequently, we did not expect to elucidate the full, true picture; nevertheless, we brought to light some important biological associations, which have motivated us to continue this research and confirm the results in a larger cohort.

Conclusions

Currently, there is a great need to develop a clinically applicable method for identifying potential susceptibility to toxicity among patients before treatment initiation. We found that patients with NM displayed upregulations of *HLA-DR1* and *HLA-DRB5* compared to patients with UM and healthy individuals. These genes encode proteins expressed on the surface of antigen presenting cells in mucosa, which suggested that the immune response might play a major role as a primary effector in UM. Indeed, the results suggested that expression of a certain isoform of *HLA-DRB1* might diminish the inflammatory response to melphalan toxicity. However, because the levels of *HLA-DRB1* and *HLA-DRB5* expression were constant throughout treatment, this isoform may serve as a predictor of UM. The findings in this study were based on a small number of samples, and thus, our results must be validated in a larger patient cohort.

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Oral mucosa tissue gene expression profiling before, during, and after radiation therapy for tonsil squamous cell carcinoma

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Abstract

Objectives: Radiation-therapy (RT) induces mucositis, a clinically challenging condition with limited prophylactic interventions and no predictive tests. In this pilot study, we applied global gene-expression analysis on serial human oral mucosa tissue and blood cells from patients with tonsil squamous cell cancer (TSCC) to identify genes involved in mucositis pathogenesis.

Materials and Methods: Eight patients with TSCC each provided consecutive buccal biopsies and blood cells before, after 7 days of RT treatment, and 20 days following RT. We monitored clinical mucositis and performed gene-expression analysis in tissue samples. We obtained control tissue from nine healthy individuals.

Results: After RT, expression was upregulated in apoptosis inducer and inhibitor genes, *EDA2R* and *MDM2*, and in *POLH*, a DNA-repair polymerase. Expression was downregulated in six members of the histone cluster family, e.g., *HIST1H3B*. Gene expression related to proliferation and differentiation was altered, including *MKI67* (downregulated), which encodes the Ki-67-proliferation marker, and *KRT16* (upregulated), which encodes keratin16. These alterations were not associated with the clinical mucositis grade. However, the expression of *LY6G6C*, which encodes a surface immunoregulatory protein, was upregulated before treatment in three cases of clinical none/mild mucositis, but not in four cases of ulcerative mucositis.

Conclusion: RT caused molecular changes related to apoptosis, DNA-damage, DNA-repair, and proliferation without a correlation to the severity of clinical mucositis. *LY6G6C* may be a potential protective biomarker for ulcerative mucositis. Based on these results, our study model of consecutive human biopsies will be useful in designing a prospective clinical validation trial to characterize molecular mucositis and identify predictive biomarkers.

Introduction

Treatment-related toxicity remains a major concern in patients with head and neck cancers [1-3], including squamous cell cancer of the tonsil (TSCC). The incidence of TSCC is increasing, due to a shift towards younger patients with human papilloma virus (HPV)-positive cancers [2,9]. Consequently, more survivors must live with both short- and long-term cancer treatment side effects, including mucositis, hypo-salivation, tissue fibrosis, and hypo-vascular bone [10,11].

For curative intentions, radiation therapy (RT) is a key modality, with or without surgery, combined with concomitant chemotherapy. Squamous cell carcinomas require a relatively large amount of radiation (60 to 70 Gray [Gy]) [4]. Recently, outcomes have improved with the advent of radio-sensitizers and intensity-modulated RT. However, mucositis remains an acute, painful side effect [5]. Mucositis appears clinically at a total dose of approximately 35 Gy (after about 2 weeks), and it gradually worsens with each dose delivered [3,6]. The incidence of mucositis is 85% in patients with head and neck cancers that require RT; of these, 37% require hospitalization, and of these 51% require a feeding tube [7,8]. The lack of predictability of who are severely affected is a significant clinical challenge [12]. Palliative interventions may relieve the side effects, but no preventive medications are available that can reduce mucositis, and no markers are available for pretreatment identification of patients likely to be severely affected [13]. Previous studies have shown that RT causes DNA damage and oxidative stress, which subsequently lead to activation of p53-induced radiotoxic pathways, apoptosis, and cell-cycle arrest [14-16]. Furthermore, DNA repair and damage response via MDM2 suppression of p53, was also reported a consequence of RT in addition to radiation fibrosis, and endothelial damage [17-18]. However, no studies have described a gene expression analysis of human mucosa [19].

Here, we describe a disease- and treatment-specific global gene expression (GGE) pilot study. We examined consecutive mucosa biopsies and peripheral blood cells collected from patients with

TSCC during RT treatment. This study aimed to generate phenotypic data to document the feasibility of a novel in vivo model of consecutive human biopsies during RT treatment that might provide new biological knowledge of the molecular pathogenesis of severe mucositis and facilitate the identification of potential predictive biomarkers.

Materials and methods

Patients

The Committee on Health Research Ethics of the Northern Denmark Region (N-20100022) approved the clinical protocol for this study. Informed written consent was obtained from all patients, in accordance with the Declaration of Helsinki. Patients were enrolled from September 1, 2010, to April 30, 2013. Inclusion criteria were age ≥18 years, cancer-treatment naïve, and without uncontrolled competitive disease.

We recruited 19 patients at the Department of Maxillofacial Surgery, Aalborg University Hospital. Among those patients, nine displayed histologically confirmed TSCC and a metastasis-negative FDG-PET/CT scan. Of these nine patients, seven provided three consecutive buccal biopsies and peripheral blood samples. The first biopsy and blood sample set (baseline) was acquired before the start of RT, the second set was acquired after one week of RT, and the third set was acquired at an average of 20 days after the last RT, for outline of the study plan (Fig 1). Two of the nine included patients died during treatment; one after the second biopsy and one before the first biopsy. The patient that died after the second biopsy was included in the analysis; thus, we analyzed eight patient samples. Our control group comprised 10 healthy, non-smoking individuals that had participated in a previous study [20]. Of these, one was excluded, due to an autoimmune disease that was not reported at the time of biopsy; thus, nine control samples were analyzed. All patients

underwent pretreatment evaluations, including a medical history, smoking habits (smokers were defined as smoking more than 10 cigarettes per day), alcohol consumption (consumers were defined as drinking more than 21 units of alcohol weekly), and a clinical examination. Baseline characteristics were noted, including age, gender, and Eastern Cooperative Oncology Group (ECOG) performance status. Before RT, patients were screened for dental infections, and when indicated, infections were eradicated.

TSCC tumors were staged according to the TNM system for staging cancer (T = size of primary tumor; N = presence and level of lymph node involvement; M = presence of distant metastasis) [21]. Immunohistochemistry was performed to detect p16 overexpression in the tumor, which indicated HPV-induced TSCC. All patients with TSCC received intention-to-cure treatments. Accelerated external RT was applied in 6 weekly fractions of 2 Gy. RT was supplemented, when indicated, with concomitant cisplatin (40 mg/m^2) once weekly during RT, according to international guidelines [22,23]. We noted the total radiation dose (Gy) applied to the tumor, based on the radiation schemes. We also calculated the estimated dose applied to the buccal mucosa at the site of the biopsy.

Mucositis assessment

Oral mucositis (OM) was evaluated weekly in all patients, by the same researcher (MM), according to the World Health Organization oral toxicity assessment worksheet [24]. Subjective symptoms (pain and ability to eat solid food) and objective oral mucositis-related findings (erythema, ulceration) were noted. Grades 0 and 1 comprised none/mild mucositis (NM); this included soreness, with or without erythema, but solid food could be taken. Grades 2 to 4 comprised ulcerative mucositis (UM); in UM, food intake gradually declined, due to pain, and parenteral feeding might become necessary.

Collection of mucosa tissue and blood cells

Sample collection was performed with the methods described previously [20]. Briefly, a lens-formed, 5-mm biopsy was harvested in a standardized manner, and the wound was tightly sutured. One half of the biopsy was immediately embedded in RNA*later*™, for GGE analysis. The other half was fixed in formalin and embedded in paraffin for immunohistochemistry.

Within 2 h of the biopsy procedure, 15 ml of EDTA-mixed venous full blood was collected. Then, mononuclear cells were isolated and stored at -196 °C in liquid nitrogen, until analysis.

Gene expression analysis

Gene expression was evaluated with the methods described previously in detail [20]. Briefly, for both mononuclear cells and mucosa, we used the Affymetrix GeneChip Human Exon 1.0 ST Arrays with the Affymetrix GeneChip WT Terminal Labeling and Controls Kit (P/N 901524). CEL files were generated with Affymetrix GeneChip Command Console Software and deposited in the NCBI Gene Expression Omnibus repository, under number GSE103412.

Immunohistochemistry

Tissue blocks were cut in 4-µm sections, and the sections were mounted on glass slides. With an in-house optimized protocol, tissues were stained for scinderin with a rabbit polyclonal antibody (KIAA1905, Nordic Biosite, www.nordicbiosite.com). Stained slides were scanned on a Hamamatsu NanoZoomer slide scanner and analyzed with NDP viewer software. To estimate the scinderin stain intensity, each stained slide was viewed at a magnification of ×15, and evaluated within a framed rectangle of 0.75 × 0.4 mm (0.3 mm^2). Samples were classified as no stain (0), lightly stained (+), or heavily stained (++).

Statistical analysis

All statistical analyses were performed with R [25] version 3.2.2 and Bioconductor packages [26].

Estimation of sample size

We applied the method described by Lee and Whitmore to identify genes that varied more than two-fold between test points, with a false discovery rate (FDR) less than 0.05% and a power of 90% [27]. This analysis was implemented in the R-package, size-power (Qui 2008). The results indicated that 10 patients in each group would provide sufficient statistical power.

Data preprocessing

The CEL files produced by the Affymetrix Expression Console were preprocessed and summarized at the gene level with the RMA algorithm in the Bioconductor package, affy, based on custom CDF-files [28]. This preprocessing step revealed the expression levels of 38,830 genes for each exon array. Genes were annotated with Ensembl gene identifiers.

Detection of differential expression

With the patient ID as a cluster variable, we used the limma package, a linear mixed model analysis provided in R, and the empirical Bayes approach to test for significant differences in gene expression between the second biopsy/blood sample and baseline, and between the third biopsy/blood sample and baseline [29]. To test for significantly differentially expressed genes between baseline and control samples, an unpaired t-test was performed with limma [29]. Patients were divided into two groups based on mucositis status (UM or NM), and significantly differentially expressed genes were detected with limma at each time point.

The FDR-adjusted P-values (≤ 0.05) were controlled with the Benjamini–Hochberg method [30] for each of the above tests.

Hierarchical clustering

The GGE data set of all nine control samples and the eight TSCC samples were divided into eight subsets. These subsets were gene biotypes (defined as protein coding), pseudogenes, miRNA, rRNA, snoRNA, snRNA, linRNA, and antisense transcript. Each dataset was subjected to hierarchical clustering, where the Pearson correlation was used as a distance measure, and average linkage was used as the algorithm method [31].

RESULTS

Clinical characteristics of TSCC

The pilot study design is shown in Fig 1. Three steps of intervention were planned during TSCC-specific standard therapy, which included RT and cisplatin treatments. We collected 32 biopsies and performed 32 blood draws (7 × 3 sample sets + 1 × 2 sample sets for TSCC and 9 × 1 sample set for controls).

Fig 1. The pilot study design. Tissue samples were collected from patients with tonsil squamous cell cancer (TSCC) at three time points: at baseline, before RT (Day0), after 7 days of RT (Day/), and 20 days after the last RT. In addition, tissue samples were collected from healthy subjects (CON). All tissue collections consisted of one blood sample and one biopsy of oral buccal mucosa. Tissue samples were successively stored in our biobank. Once all the material was collected, gene expression analysis was performed collectively at the same laboratory.

Patient clinical characteristics and demographics are shown in Table 1. Age was comparable between the control (age 58 years, range 47–78) and TSCC (age 63.5 years, range 51–69) groups. A trend towards male dominance was observed in the TSCC group (2 females among 8 patients), but not in the control group (4 females among 9 patients). Five of eight patients with TSCC were smokers, and four of the eight consumed alcohol. Tumor staging was evaluated according to the TMN system [21], and p16 overexpression was detected in six of eight tumors.

Table 1. **Patient characteristics and demographics upon enrollment in the study**

Patient	**Age**	**Gender**	**ECOG[a]**	**Smoking[b]**	**Alcohol[c]**	**Staging[d]**	**p16[e]**
TSCC01	57	m	0	0	0	T1N2bM0	yes
TSCC03	67	f	2	1	1	T1N0M0	no
TSCC04	74	m	2	0	1	T1NxM0	no
TSCC05	72	m	0	0	0	T1N2aM0	yes
TSCC06	65	m	0	1	1	T1N1M0	yes
TSCC07	59	m	1	1	1	T2N1M0	yes
TSCC08	68	m	0	1	0	T4aN2cM0	yes
TSCC09	56	f	0	1	0	T2N2cM0	yes

[a]Eastern Cooperative Oncology Group (ECOG) performance status at baseline; [b]Smoking categories: 0 = Non-smoker, 1 = smoked more than 10 cigarettes per day; [c]Alcohol categories: 0 = No alcohol consumption, 1 = consumed more than 3 units of alcohol per day; [d]TNM system for staging of cancer: T = size of primary tumor; N = presence and level of lymph node involvement; M = presence of distant metastasis (1); [e]Overexpression of p16 indicates positive for HPV

Table 2. Patient clinical data during radiation treatment and at follow-up

Patient	**Total dose of radiation to tumor**	**Estimated dose of radiation at biopsy site**	**Cisplatin (40 mg/m^2) once weekly during RT**	**WHO[a]**	**Days from treatment start to second biopsy**	**Days from second to third biopsy**

TSCC01	66 Gy/33 fr	4.2	yes	3	3	53
TSCC03	66 Gy/33 fr	9.5	yes	0	8	42
TSCC04	68 Gy/34 fr	9.3	yes	1	8	dead
TSCC05	68 Gy/33 fr	14.4	yes	2	12	41
TSCC06	66 Gy/33 fr	5.3	no	1	4	40
TSCC07	68 Gy/34 fr	5.1	yes	3	4	52
TSCC08	76 Gy/56 fr	6.9	no	2	9	80
TSCC09	68 Gy/34 fr	7.2	yes	3	6	92

Abbreviations: Gy: Gray; fr: fractionated; WHO: World Health Organization; [a]mucositis stage, according to the WHO assessment scale, was measured weekly, during treatment and after, until mucositis dissolved [24].

The clinical data collected during RT are shown in Table 2. After treatment initiation, the second biopsy was acquired at a median of 7 days (range 3–12), and the third biopsy was acquired at an average of 57 days (range 40–92). Two patients, TSCC06 and TSCC08, did not receive cisplatin. An average dose of 68.3 Gy (range 66–76) was applied to the tumors. According to the radiation schemes, a total dose of approximately 30–35 Gy was applied to the buccal mucosa bilaterally. At the time of the second biopsy, an average dose of 7.7 Gy (range 4.2–14.4) was applied. UM was detected in five patients and NM was detected in three patients. The median mucositis scores were 1.9 (range 0–3): 2.6 for the UM group and 0.7 for the NM group. We observed no signs of infection at the site of biopsy. All samples yielded valid gene expression profiles.

Gene expression in mucosa and blood

The differentially expressed genes in mucosa (Table 3) and blood (Table 4) are annotated with a gene symbol, the fold change (FC), the adjusted p-value, the gene ontology terms (GO-terms), and the gene function.

Table 3 Genes altered in mucosal tissue from patients with tonsil squamous cell carcinoma receiving radiation therapy

Gene symbol	FC	p-value	adj. p-value	Qualified Gene Onotology term	Function
Baseline					
LIFR	-2.73	2.09e-05	0.019	Leukemia Inhibitory Factor Receptor Alpha	Cellular differentiation, proliferation, survival
FKBP5	-2.48	0.00015	0.037	FK506 Binding Protein 5	Immune regulation, basic cellular processes
SPARCL1	-2.24	0.0002	0.041	SPARC Like 1	Cell adhesion, migration, and proliferation
MS4A4E	-2.30	9.06e-06	0.018	Membrane Spanning 4-Domains A4E	Cell surface signaling
PDGFRA	-2.11	1.74e-06	0.010	Platelet Derived Growth Factor Receptor Alpha	Cell surface tyrosine kinase receptor
RN7SL783P	2.54	0.00010	0.031	pseudogene	Unknown function
MTND5P8	2.17	0.0002	0.04	pseudogene	Unknown function
ABO	2.02	8.82e-07	0.001	Alpha 1-3-N-Acetylgalactosaminyltransferase	Enzyme, modifying surface glycoproteins
After seven days of radiotherapy					
HIST1H3B	-2.91	7.52e-08	0.000143	Histone Cluster 1, H3b	Transcription

HIST1H2BM	-2.75	1.6e-07	0.000251	Histone Cluster 1, H2bm	Transcription
CYSLTR1	-2.54	3.91e-05	0.0098	Cysteinyl Leukotriene Receptor 1	Cell structure
HIST1H3C	-2.39	9.08e-06	0.0039	Histone Cluster 1, H3c	Transcription
HIST1H3H	-2.17	4.53e-08	0.000105	Histone Cluster 1, H3h	Transcription
MOXD1	-2.16	6.19e-08	0.000128	Monooxygenase DBH Like 1	Metabolism
HIST1H1A	-2.12	0.00016	0.022	Histone Cluster 1, H1a	Transcription
HIST1H1B	-2.09	1.05e-08	3.14e-05	Histone Cluster 1, H1b	Transcription
MKI67	-2.00	2.58e-06	0.0016	Marker Of Proliferation Ki-67	Transcription
WDR63	2.67	1.09e-10	1.1e-06	WD Repeat Domain 63	Unknown
MDM2	2.29	6.77e-11	4.26e-11	MDM2 oncogene, E3 ubiquitin protein ligase	Apoptosis
EDA2R	2.26	8.38e-11	1.0e-06	Ectodysplasin A2 receptor	Apoptosis
POLH	2.17	3.22e-10	1.81e-06	Polymerase; DNA directed	Transcription
KRT16	2.15	0.00058	0.052	Keratin 16	Cell structure
Three weeks after RT cessation					
ANKRD20A5P	-3.56	2.90e-07	0.0026	Ankyrin Repeat Domain 20 Family Member A5	Pseudogene
CYSLTR1	-3.11	3.92e-06	0.0082	Cysteinyl Leukotriene Receptor 1	Cell structure
SCIN	-2.50	9.09e-05	0.044	Scinderin	Cell structure
ANKRD20A11P	-2.47	4.93e-05	0.033	Ankyrin Repeat Domain 20 Family Member A11	Pseudogene
ANKRD20A9P	-2.32	1.2e-06	0.0052	Ankyrin Repeat Domain 20 Family Member A9	Pseudogene
CYP4F34P	-2.28	4.1e-05	0.032	Cytochrome P450 Family 4 Subfamily F Member 34	Pseudogene
TC2N	-2.13	6.47e-05	0.036	Tandem C2 Domains, Nuclear	Metabolism
IL1R2	-2.12	3.37e-07	0.0026	Interleukin 1 Receptor Type 2; cytokine receptor of the interleukin 1 receptor family	Immune response
MIR31HG	5.30	5.71e-05	0.035	Non-coding microRNA no 3	Non-coding mi-RNA

CCAT1	3.08	1.08e-05	0.018	Colon Cancer Associated Transcript 1	Non-coding RNA
PTPRZ1	2.93	0.000103	0.047	Protein Tyrosine Phosphatase, Receptor Type Z1	Transcription
LY6G6C	-3.78	2.53e-06	0.0995	Lymphocyte Antigen-6 G6C	Signal transduction Immune response

Table 4 Genes altered in mononuclear cells of the blood from patients with tonsil squamous cell carcinoma receiving radiation therapy.

Gene symbol	**FC**	**p-value**	**adj. p-value**	**Qualified Gene Onotology term**	**Function**
Baseline					
RNU6-620P	-11.8	1.48e-12	5.80e-08	RNA, U6 small nuclear 620, pseudogene	pseudogene
RNU6-422P	-3.77	3.03e-08	0.00022	RNA, U6 small nuclear 422, pseudogene	pseudogene
RNU6-737P	-3.36	1.34e-07	0.00034	RNA, U6 small nuclear 737, pseudogene	pseudogene
RNU6-795P	-2.85	2.82e-06	0.0024	RNA, U6 small nuclear 795, pseudogene	pseudogene
RPS7P2	-2.63	2.14e-07	0.00044	Ribosomal protein S7 pseudogene 2	pseudogene
AGAP9	-2.61	6.15e-06	0.0039	ArfGAP With GTPase Domain, Ankyrin Repeat And PH Domain 9	GTPase-activating
RNU6-336P	-2.45	5.72e-08	0.00025	RNA, U6 small nuclear 336, pseudogene	pseudogene
OAZ1	-2.26	6.81e-06	0.0040	Ornithine decarboxylase antienzyme 1	Cell growth and proliferation
RPL23AP64	-2.19	0.00012	0.018	Ribosomal protein L23a pseudogene 64	pseudogene
RNU6-1162P	-2.06	2.02e-05	0.0068	RNA, U6 small nuclear 1162, pseudogene	pseudogene
CCDC144B	-2.02	0.00074	0.043	Coiled-Coil Domain Containing 144B	pseudogene
RN7SL432P	-2.02	5.37e-07	0.00088	RNA, 7SL, cytoplasmic 432, pseudogene	pseudogene
RNU6-622P	7.30	7.74e-09	8.62e-05	RNA, U6 Small Nuclear 622, Pseudogene	pseudogene
DUTP6	3.45	1.74e-06	0.0019	Deoxyuridine Triphosphatase Pseudogene 6	pseudogene

SSU72P8	3.44	1.07e-07	0.0014	RNA Polymerase II CTD Phosphatase Homolog, Pseudogene 8	pseudogene
RNU6-919P	3.37	1.06e-05	0.0051	RNA, U6 Small Nuclear 919, Pseudogene	pseudogene
RPS6P15	3.01	2.82e-06	0.0024	Ribosomal Protein S6 Pseudogene 15	pseudogene
RN7SL748P	2.44	1.59e-05	0.0061	RNA, 7SL, Cytoplasmic 748, Pseudogene	pseudogene
RPL10P4	2.33	2.88e-07	0.00051	Ribosomal Protein L10 Pseudogene 4	pseudogene
RPL21P133	2.32	6.39e-07	0.0010	Ribosomal Protein L21 Pseudogene 133	pseudogene
RN7SL290P	2.22	1.06e-05	0.0051	RNA, 7SL, Cytoplasmic 290, Pseudogene	pseudogene
OR5M4P	2.21	4.97e-05	0.011	Olfactory Receptor Family 5 Subfamily M Member 4 Pseudogene	pseudogene
RNU6-151P	2.19	1.58e-07	0.00036	RNA, U6 Small Nuclear 151, Pseudogene	pseudogene
RNU6-135P	2.19	1.29e-07	0.00034	RNA, U6 Small Nuclear 135, Pseudogene	pseudogene
RNA5SP116	2.18	0.00085	0.046	RNA, 5S Ribosomal Pseudogene 116	pseudogene
NUTM2D	2.13	0.00016	0.021	NUT family member 2D	unknown
RNA5SP54	2.06	8.49e-08	0.00030	RNA, 5S Ribosomal Pseudogene 54	pseudogene
RN7SL865P	2.05	0.00074	0.043	RNA, 7SL, Cytoplasmic 865, Pseudogene	pseudogene
RPS29P8	2.00	8.69e-07	0.0012	Ribosomal Protein S29 Pseudogene 8	pseudogene

Before RT was applied, eight genes were altered in patients with TSCC compared to controls (Table 3). Five of these genes remained unaffected with subsequent therapy: *LIFR*, *FKBP5*, *SPARCL1*, *MS4A4E*, and *PDGFRA*.

In response to treatment, we found nine downregulated genes. Eight of these genes were in the histone cluster family, including *HIST1H3B*, *HIST1H2BM*, *HIST1H3C*, *HIST1H3H*, *HIST1H1A*, and *HIST1H1B*; and one, *MKI67*, was a marker of Ki-67 proliferation. Five genes were upregulated. Of these, two were related to apoptosis, *MDM2* and *EDA2R*, and one, *POLH*, encoded a transcriptional DNA-directed polymerase (Table 3).

On day 21 after the last RT application, we found 11 altered genes compared to baseline (Table 3). Most were pseudogenes, including *ANKRD20A5P*, *ANKRD20A11P*, *ANKRD20A9P*, and

CYP4F34P (downregulated); and noncoding RNAs, *CCAT1* and *MIR31HG*. The *MIR31HG* RNA was upregulated only among patients with TSCC that received cisplatin. *IL1R2* (downregulated) encoded a cytokine receptor of the interleukin 1 receptor family. *SCIN* (downregulated) encoded a regulatory protein involved in exocytosis. Immunohistochemical analysis results (Fig 2) showed reduced expression in epithelial cells, but not in the salivary glands.

Fig 2. Immunohistochemical analysis of mucosal tissue expression of scinderin. Oral buccal mucosa section (×15 magnification) stained with an *SCIN* antibody. (a) High scinderin expression is evident in mucosa from a control individual (patient CON05). (b) Low scinderin expression is evident in mucosa from a patient with tonsil squamous cell cancer (patient TSCC07); the biopsy was acquired Day20. *SCIN* encodes a regulatory protein involved in exocytosis and we expected to se downregulation in salivary gland tissue, however epithelial cells were heavily stained in the healthy control group.

When gene expression profiles of the buccal mucosa were compared between UM and NM samples, we found no differentially expressed genes (adjusted $P < 0.05$) in either blood or mucosa. However, one gene, *LY6G6C*, tended to be expressed at low levels (FC -3.78; adj. $P = 0.0995$) in UM baseline biopsies **(**Fig 3, Table 3).

Fig 3. Expression of *LY6G6C* in mucosa. Dot plot shows expression of *LY6G6C*, at baseline (Day 0), after 7 days of RT (Day 7), and 20 days after the last RT session (Day 20), among patients that developed ulcerative mucositis (red) or mild/no mucositis (green), and in controls (blue). Patients with mild/ no mucositis exhibited an upregulation of *LY6G6C*. *LY6G6C* encodes a surface immunoregulatory protein expressed on mucosal dendritic cells.

We found 12 downregulated and 17 upregulated genes in blood samples from the TSCC group compared to the control group (Table 4). These genes were dominated by small nuclear RNAs (snRNAs), e.g., *RNU6-620P* and *RNU6-622P*, and a dot plot of these two selected genes is shown in Fig 4.

Fig 4. Expression of *RNU6-620P* and *RNU6-622P* in blood cells. Dot plot shows expression of *RNU6-620P* (FC-x11.8; P=5.80e-80) and *RNU6-622P* (FCx7.3 P=8.62e-05) at baseline (Day 0), after 7 days of RT (Day 7), and 20 days after the last RT session (Day 20), among patients that developed ulcerative mucositis (red) or mild/no mucositis (green). Expression in normal controls is indicated with blue circles. These genes encode small nuclear RNAs, which are non-protein coding genes. Patients with TSCC expressed a significant different level of both genes compared to healthy controls.

We clustered the expression levels of snRNAs, regardless of fold-changes according to P-value, and observed a distinct division between patients with TSCC and healthy controls (Fig 5). We found no differentially expressed genes in blood samples between baseline and day 7 or day 20.

Fig 5. Small nuclear RNA (snRNA) clustered according to P value. The expression of snRNA regardless of fold change was clustered according to P value, showing a distinct division between patients with TSCC and healthy controls.

Discussion

This study aimed to validate our clinical pilot study set-up and demonstrate its potential for identifying pathogenic variables or biomarkers for molecular mucositis. In response to RT, we identified several altered genes in the mucosa, but no differentially expressed genes in the blood cells. Furthermore, the identified genes were not correlated to the grade of clinical mucositis. Furthermore, we found that although all patients with TSCC were diagnosed with a localized solid epithelial tumor, and the biopsies from the study group was harvested from clinically healthy buccal mucosa, the mucosal tissue and blood cells had different gene profiles compared to healthy controls before RT.

RT effects on mucosal gene expression

Several studies have described the molecular effects of radiation on normal tissue, but no studies have described effects on gene expression levels [19]. Generally, short-term alterations include increased levels of p53 and other apoptotic markers (e.g., Bcl-2 and Mcl-1) [14,32], increased numbers of inflammatory cells (CD68-positive macrophages and other leukocyte subtypes), and alterations in inflammatory markers (e.g., NF-kB and COX-2) [32-35]. The epithelium begins to regenerate after one week of radiation, confirmed by the identification of cell proliferation markers, Ki-67 and [^{3}H]-TdR , and by the increased expression of keratins (keratins 1, 6, 10, 16) [37]. Over the long term, RT caused different distribution patterns of adhesion molecules and macrophage subpopulations, compared to pretreatment specimens [38].

The present pilot study identified markers of apoptotic activity. *EDA2R* was upregulated in the mucosa 7 days after RT initiation, compared to its pretreatment status. *EDA2R* encodes the ectodysplasin A2 receptor, a transmembrane protein in the tumor necrosis factor receptor superfamily. Upon stimulation, this receptor activates the NF-κB and JNK apoptotic pathways . In addition, six members of the histone cluster family were downregulated, which indicated DNA damage. Histones are basic nuclear proteins responsible for nucleosome structure. Previous studies in cell lines have described histone downregulation in response to RT [40].

In parallel, the *MDM2* oncogene (*MDM2*) was upregulated. *MDM2* encodes an E3 ubiquitin ligase, localized in the nucleus, and is regulated transcriptionally by p53. In turn, E3 ubiquitin ligase mediates the ubiquitination of p53, and thereby, inhibits p53-mediated cell-cycle arrest and apoptosis [41]. In addition, the upregulation of *POLH*, a polymerase that replicates UV-damaged DNA, indicated a DNA defense mechanism. Thus, we identified both inducers and inhibitors of apoptosis and DNA damage, consistent with findings reported in previous preclinical studies.

Proliferation-related genes were also altered. *MKI67*, which encodes Ki-67, a nuclear protein that is essential for cellular proliferation, was downregulated after 7 days of RT. This finding contrasted with findings from a previous study on human mucosa [32]. However, *KRT16* was upregulated. *KRT16* encodes keratin16, a protein characteristic of early differentiated epithelial cells. This short-term change indicated continuous epithelial proliferation [42]. This finding was also reported in a previous study [37].

SCIN encodes a calcium ion- and actin filament-binding protein with a regulatory function in exocytosis [43]. We expected *SCIN* to be associated with salivary gland function because of the connection to exocytosis function and prior studies have reported histological changes in radiated salivary glands, including atrophy, edema, cell death, and fibrous tissue formation [44]. However an

immune histochemical stain for SCIN revealed that the presence was seen in the epithelial cells (Fig 2).

A potential biomarker for the grade of clinical mucositis

When we compared samples from three patients with NM to samples from 4 patients with UM, we found that *LY6G6C* (lymphocyte antigen 6 complex, locus G6C) was upregulated (x3.78; P=0.0995) in NM before treatment, although this finding was not statistically significant (Fig 3). *LY6G6C* belongs to a cluster of leukocyte antigen-6 genes linked to the major histocompatibility complex–class II. This complex is located at the cell surface, where it is involved in immune-mediated signal transduction.

In a previous study, we showed that two members of the major histocompatibility complex–class II gene family, *HLADR-B1* and *B5*, could potentially predict UM in patients with multiple myeloma [20]. We therefore hypothesize that HLA-based immunity protect against tissue inflammation during treatment in both these disease categories. Because mucositis may be considered an inflammatory state, those findings might add to our molecular understanding of why RT induces severe mucositis in some patients.

Pretreatment gene signature of TSCC

In mucosa, we found that TSCC induced a specific gene signature different from controls (Table 3). In particular, we found that TSCC induced changes in the expression of leukemia inhibitor factor receptor-alpha (*LIFR*), platelet-derived growth factor receptor-alpha (*PDGFR*), and secreted protein acidic and cysteine rich-like protein (*SPARCL*) genes. First, this signature was present in clinically normal-appearing oral mucosa located at a distance from the tonsil tumor. Second, the signature was expressed independently of alcohol consumption, smoking habits, and p16 overexpression in

the tumor, in addition to other clinical features. Third, this signature remained practically unchanged throughout RT.

LIFR, a transmembrane receptor protein of the type 1 cytokine receptor family, is involved in cellular differentiation, proliferation, and survival; moreover, it inhibits the p53 apoptotic pathway. Low expression has been detected in various human cancers [45]. However, *LIFR* has been identified as both a suppressor and a promoter of carcinogenesis. *PDGFR* encodes a cell-surface tyrosine kinase receptor that binds platelet-derived growth factor family members. The receptor complex activates pathways involved in cell migration and chemotaxis during wound healing [46]; additionally, mutations in *PDGFRA* play an active role in cancer development [47]. Finally, *SPARCL* is involved in extracellular matrix synthesis. It was downregulated in number human cancer types [48]. It remains unclear why these genes, which are involved in cellular differentiation, wound healing, and extracellular matrix formation, are downregulated in clinically normal-appearing mucosa acquired from patients with TSCC. Future studies should investigate whether this phenotype might indicate increased susceptibility to malignant transformation.

The GGE analysis of blood samples revealed a large array of snRNA-type pseudogenes. Of these, *RNU6-620P* was downregulated 11.8-fold (P=5.80e-80) and *RNU6-622P* was upregulated 7.3-fold (P=8.62e-05) compared to controls. A cluster analysis of the expression of snRNAs and other noncoding RNAs in the blood revealed that distinctly different clusters of noncoding RNAs were associated with TSCC and controls (Fig 5). The protein coding genes did not show the same distinction.

CONCLUSION

In this pilot study, we described a gene signature expressed by mucosal tissue and circulating peripheral blood cells from patients with TSCC in response to RT. RT caused molecular alterations

related to apoptosis, DNA damage, DNA repair, and proliferation. However, these alterations were independent of clinical mucositis severity. Furthermore we identified a potential protective biomarker for ulcerative mucositis. Based on these results, we concluded that our model was feasible, and the data will be useful in designing a prospective clinical validation trial for characterizing mucositis at the molecular level and identifying predictive biomarkers.

ACCESION CODES

Phenotype data described in this manuscript was deposited at the NCBI Gene Expression Omnibus (GEO) repository under no GSE103412.

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MOLECULAR CHARACTERIZATION OF MUCOSA IN PATIENTS WITH ACUTE MYELOID LEUKEMIA.

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ABSTRACT

Background: Mucositis is a frequent clinical complication to chemotherapy among patients with acute myeloid leukaemia (AML), however, the molecular mechanisms are not fully understood. Here our first goal is to present molecular data and conclusions from a pilot study of global gene expression (GGE) in human mucosa biopsies before, during and after induction treatment for AML. Our second goal is to compare the mucosa tissue GGE profile of AML patients with multiple myeloma (MM), and tonsil squamous cell carcinoma (TSCC) profiles.

Materials and Methods: Six of nine primary included de novo AML patients delivered three consecutive buccal biopsies for analysis: before ADE (Cytarabine/Daunorubicin/Etoposide) induction therapy (day0), day 3 of treatment (day3) and before initiation of second induction therapy (day27). Buccal biopsies from nine healthy individuals served as controls (CON). Patient's clinical characteristics including mucositis assessment were registered and correlated to gene expression.

Results: Before treatment, *RNU6-996P*, a non-coding small nuclear RNA, was significant upregulated in AML compared to normal CON mucosa. In response to chemotherapy, genes with DNA repair, transcription and cell growth regulation were altered: *POLH, HIST1H1*, *HIST1H2BM*; and *NOTCH1*. Most important principal component analysis of pre-treatment GGE revealed differences between MM and AML or TSCC mucosa tissues.

Conclusions: Therapy-induced molecular changes in the mucosa of patients with AML were dominated by DNA damage and repair genes. Most important the mucosal gene expression from three independent cancer cohorts identified disease specific differences.

INTRODUCTION

Mucositis is a dose reducing side effect in the treatment of patients with acute myeloid leukaemia (AML), a blood cancer characterized by accumulation of abnormal blasts in the marrow that interfere with normal haematopoiesis and infiltrate the blood with immature blasts [1,2]. One year survival rate is around 70 % for patients less than 60 years, and far less, approximately 50 % for older patients following induction therapy [1]. The standard treatment of AML consists of a combination of Cytarabine, Daunorubicin, and Etoposide (ADE induction treatment). Often severe side effects associated to mucositis shorten or decrease planned treatment intensity. To reduce oral symptoms and febrile episodes during treatment, removal of inflammatory oral conditions prior to cancer treatment was recognized early in this patient group [3,4] in addition to prophylaxis towards reactivation of virus or opportunistic bacteria [4,5].

The molecular mechanisms causing the diversity in clinical expression of mucositis during cancer treatment, has been studied in animal models and only few studies on the molecular changes during cancer therapy in human mucosa exists [6]. Preventive agents or interventions that have proven well when tested in animal models, fails to show any benefits in the clinic. At present, international recommendations for prophylaxis and treatment of mucositis are few and consist primarily of palliation of pain and infection control [7].

In the present pilot study, we aimed to identify initial changes in gene expression in mucosa biopsies from a cohort of patients with AML during ADE induction treatment. Furthermore, we wanted to compare pre-treatment mucosa gene expression profiling in patients with AML with two earlier comparable studies on patients with multiple myeloma (MM) and tonsil squamous cell carcinoma (TSCC) [8, 19].

MATERIALS AND METHODS

Patients

We recruited 9 patients with AML at age 18 or above, cancer treatment naïve and without uncontrolled additional disease, from the Haematological Department, Aalborg University Hospital, Denmark from September 1st, 2010 to April 30st 2013. We also included 9 healthy, non-smoking, individuals as controls (CON) [8]. Of the nine patients with AML, five patients had all three biopsies taken, and one patient only 2 biopsies after which the consent was withdrawn. One patient was referred to intensive care after the first biopsy and was removed from the protocol for ethical reasons. Two patients withdrew their consent after the first biopsy. This resulted in six eligible patients with AML for the planned molecular mucosa studies.

The first biopsy was obtained immediately before start of induction therapy (day0); the second biopsy was obtained after 3-4 days of therapy (day3); and the third was obtained 19-47 days after the last chemotherapy during an outpatient control visit (day21). A detailed study plan is shown in **Figure 1**. The clinical protocol was approved by The Committee on Health Research Ethics of Northern Denmark (**N-20100022)** and an informed written consent was obtained from all included patients in accordance with the Declaration of Helsinki.

All patients underwent full initial medical evaluation including medical history and clinical examination, and characteristics including age, gender, Eastern Cooperative Oncology Group (ECOG) performance status at baseline, smoking habits (smoking more than 10 cigarettes per day), alcohol consumption (drinking more than 21 units of alcohol weekly), were registered. Patients were screened for dental infections and, if indicated, these infections were removed prior to chemotherapy. AML patients received Cytarabine (100mg/m^2) and Daunorubicin (50mg/m^2) for 5 respectively 2 days if age 70 or above, and if age less than 70, for 10 and 3 days supplied with

Etoposide (100mg/m^2) day 1, 3 and 5, for details on treatment see **Table 1**. Patients received antiviral, antifungal and antibacterial treatment according to department protocols. The clinical characteristics and demographics of the patients with AML appear in **Table 1**.

Mucositis assessment

Daily, during hospital admission, oral mucositis (OM) status was recorded according to WHO oral toxicity assessment worksheet (Quinn 2007), which included registration of subjective symptoms (ability to eat solid food and pain score) and objective findings of mucositis signs (erythema or ulceration). Grades 0 and 1 indicated none or very mild mucositis (NM) and grades 2-4 indicated ulceration and pain of increasing severity (UM).

Collection of mucosa

We refer to the methods used in our previous work [8]. In short, a lens-formed 5-mm biopsy was harvested in a standardized manner and the wound tightly sutured. One half of the biopsy was secured for GGE and immediately embedded in RNA*later*™, and the other half was fixated and embedded in paraffin for histochemical staining.

Gene expression profiling (GEP) procedure

We refer to a detailed description of the methods used in our previous study [8]. In brief, for analysing mucosa, we used the Affymetrix GeneChip Human Exon 1.0 ST Arrays with the Affymetrix GeneChip WT Terminal Labeling and Controls Kit (P/N 901524). CEL files were generated with Affymetrix GeneChip Command Console Software and deposited at the NCBI Gene Expression Omnibus repository, under number **GSExxxxx** (to be uploaded).

Statistical analysis

All statistical analyses were performed using R [10] version 3.2.2 and Bioconductor packages [11].

Estimation of power size: To identify genes that varied more than two fold between test points with a false discovery rate (FDR) of less than 0.05% and a power of 90%, we applied the method described by Lee and Whitmore [12] implemented in the R-package size-power (Qui 2008). The results indicated that 10 patients in each group was sufficient.

Data pre-processing: The CEL files produced by the Affymetrix Expression Console were pre-processed and summarized at the gene level using the RMA algorithm with the Bioconductor package affy using custom CDF-files [13]. This pre-processing resulted in the expression levels of 38,830 genes for each exon array annotated with Ensembl gene identifiers.

Detection of differential expression: With patient ID as a cluster variable, using the limma-package, a mixed linear model and empirical Bayes approach were performed to test for significant differences in gene expression between the second biopsy/blood test and baseline, and between the third biopsy/blood test and baseline [14]. To test for significantly differentially expressed genes between baseline and CON, an unpaired t-test was performed using limma. Patients additionally were divided into two groups based on mucositis status (UM or NM), and significantly differentially expressed genes between the two groups also were detected using limma for each time point.

The FDR-adjusted P values were controlled using the Benjamini–Hochberg method [15] for each of the above tests. Adjusted P values of 0.05 and below were considered significant.

RESULTS

Clinical characteristics

The clinical characteristics and demographics of the included patients are shown in **Table 1**. The AML and CON groups were comparable in age (AML: 65.5 years (range 58-75); CON: 58 years (range 47–78) and gender (AML: female 3/6; CON 4/9). Two of six AML patients were smokers, not any of the AML patients consumed more than 21 units of alcohol weekly. AML subtype according to the FAB classification (French-American-British classification of AML) [16] was registered: two M2, two M4, one M5 and one M6 . ECOG (1.2 (range 1-2), BMI (25.4 (range 21.4-29.3). Finally, the individual treatment was noted.

The clinical data during treatment, including mucositis assessment is shown in **Table 2**. The patients experienced clinical mucositis with an average score of 1.3 (range 0-3) and two patients experienced ulcerative mucositis. One patient responded completely while five relapsed, of these only one survived. Overall survival was 2.7 yrs. (range 1.5-4.3 yrs.); for the UM group 3.9 yrs. (range 3.4-4.3 yrs.) and for the NM group 1.9 yrs. (range 1.5-4.2 yrs.). Event free survival for the whole group was 1.1 yrs. (range 0-4.0 yrs.); for the UM group 2.9 yrs. (range 1.5-4.3 yrs.) and for the NM group 0.3 yrs. (range 0-1 yrs.).

Gene expression analysis of the mucosa samples

All biopsies produced evaluable gene profiles. Pre-treatment, two genes were differently expressed RNU6-996P and LINC01975 were significant upregulated in AML compared to normal CON mucosa.

In response to treatment, we found four genes differently expressed: two genes of the histone cluster family, *HIST1H1A* and *HIST1H2BM*, were downregulated, *POLH* encoding a transcriptional DNA

directed polymerase and *NOTCH* encoding a membrane protein responsible for intercellular signalling that regulates interactions between physically adjacent cells, were upregulated (**Figure 2**, **Table 3**)

As illustrated in **Figure 3** the gene expression of *LINC01975* (left) and *RNU6-996P* (right) before (Day0), after 3-4 days of chemotherapy (Day3), and before initiation of the second induction therapy (Day27) indicate a potential UM predictive biomarker.

Pre-treatment gene profiling in AML compared to independent cohorts of MM and TSCC

We merged expression data from two cohorts of mucosa from patients with multiple myeloma (MM) [8], tonsil squamous cell cancer (TSCC) [19] and the AML cohort to perform a principal components analysis as illustrated in **Figure 4**. Each patient delivered three consecutive biopsies before, during and after therapy. Patients cluster according to disease indicating that disease is the major contributor to the variation in gene expression of the mucosa samples and not the treatment or grade of mucositis.

DISCUSSION

AML is a heterogonous cancer of the blood with subtype classification and a moderate survival rate [1,16]. In previous similar pilot-study set-up on patients with multiple myeloma respectively with tonsil squamous cell cancer, we identified biomarkers for severe mucositis and identified a range of upregulated genes of the apoptotic and inflammatory pathways in response to treatment. In this study, only a few genes were differentially expressed in response to treatment. This may be due to the heterogeneous treatment regimen: to patients received only Cytarabine (an antimetabolic agent) and Daunorubicin (a topoisomerase II inhibitor), two patients received additional Etoposide

(also a topoisomerase II inhibitor); one patient received both Cytarabine, Daunorubicin and Mylotarg (a monoclonal antibody against CD33) and one patient received all four entities. At the time of the second biopsy patients had revived an average of 3.5 days of treatment (range 3-5) involving the above-mentioned drugs. Of the genes identified, *POLH* encodes a specialized DNA polymerase that accurately replicates UV-damaged DNA was also upregulated in the MM and TSCC group. Members of the histone cluster family were downregulated, this was also seen in the TSCC cohort. *NOTCH1* (upregulated) a cell membrane protein involved in cell signalling between adjacent cells was only upregulated in the AML cohort. *POLH* and *NOTCH1* seem to be more upregulated in the non-mucositis group as illustrated in Fig2; this tendency was not obvious in TSCC and MM cohort.

Previously only one study applied gene expression to human tissue harvested from only three patients with AML [18]. Clinical data on mucositis was collected, however this information was not correlated to the microarray data. Nevertheless, in response to therapy, eight significantly altered genes was identified; among these, the gene that encodes argininosuccinate synthase 1 (*ASS1*) was three-fold downregulated; argininosuccinate synthase 1 suppresses nitric oxide production, and consequently, promotes p53-mediated apoptosis. Moreover, they found that a gene encoding a zinc transporter (*SLC39A6*) was three-fold upregulated. The zinc transporter is involved in the epithelial-mesenchymal transition and tissue repair. We did not identify similar genes in our material.

This pilot study has together with our two other cohorts shown that disease specific transcriptomes exist has generated hypothesis that mucositis at the molecular level is heterogeneous and dependent on disease although similarities exists.

CONCLUSION

Therapy-induced molecular changes in the mucosa of patients with AML was associated by DNA damage and defence genes. A PCA analysis of mucosal gene expression from three independent cancer cohorts showed that the major contributor to the variation in gene expression of the mucosa samples were disease specific and not mucositis grade.

TABLE AND LEGENDS

Table 1. patients characteristics and demography at entrance to the study

Patient	Age	Gender	ECOG[a]	BMI[b]	Smo[c]	Alc[d]	FAB[e]	Treatment
AML01	58	m	1	29.3	0	0	M6	Cy 10 days Da 3 times Mylo
AML02	69	m	1	26.8	1	0	M2	Cy 8 days Da 3 times Etop 4 days
AML04	59	f	1	21.4	0	0	M5	Cy 8 days Da 3 times Etop 4 days Mylo
AML05	58	f	1	23.8	0	0	M4	Cy 10 days Da 3 times Etop 5 days
AML07	75	f	1	27.1	0	0	M2	Cy 5 days Da 2 times
AML09	74	m	2	23.9	1	0	M4	Cy 5 days Da 2 times

Abbreviations: a=Eastern Cooperative Oncology Group performance status; b=body mass index; c=smoking more than 10 cigarettes per day; d=drinking more than 21 units of alcohol weekly; e=FAB: French-American-British subtype classification; Cy = Cytarabine 100mg/m^2 twice daily; Da = Daunorubicin 50mg/m^2 once every second day; Etop = Etoposide 100mg/m2 once daily. Mylo = Mylotarg 3mg/m^2 once.

Table 2. Patients clinical data during chemotherapy

	[a]Mucositis grade	[b]Time b2	[c]Time b3	Relapse	[d]Overall survival	[e]Event free survival	Status at follow up
Patients with ulcerative mucositis							
AML01	3	3	41	MDS AML	3.4	1.5	dead
AML02	3	4	19	no	4.3	4.3	CR
Patients with no/mild mucositis							
AML04	0	4	No 3rd biopsy	AML	4.2	1.0	relapse
AML05	0	3	22	AML	1.5	0	dead
AML07	1	3	27	AML	1.5	0	dead
AML09	1	5	27	AML	1.5	0	dead

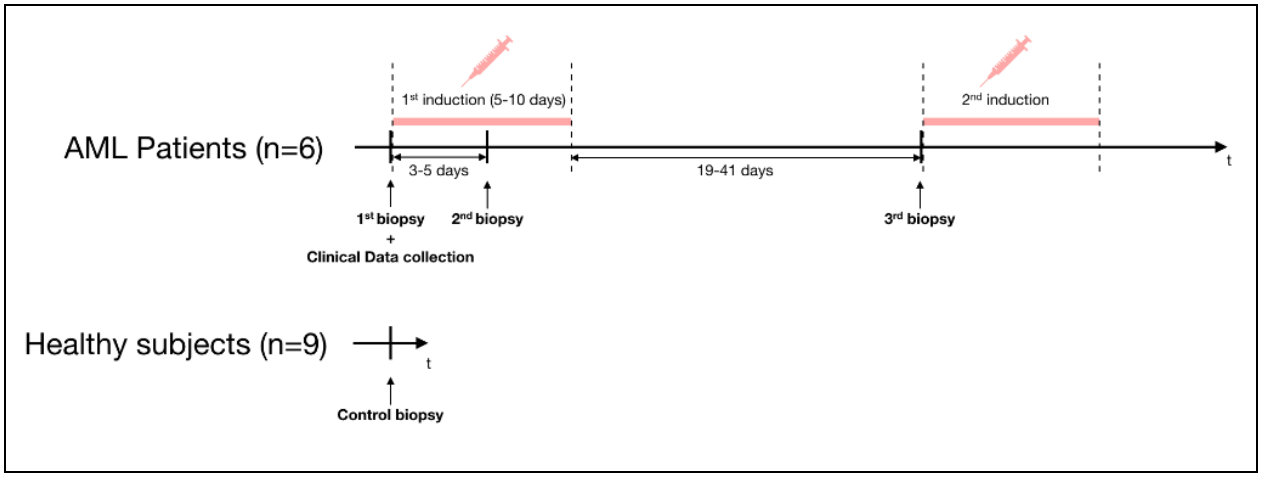
Abbreviations: a= Mucositis estimated according to WHO [17]; b=number of days from initiation of induction therapy to second biopsy; c= number of days from end of first induction therapy to third biopsy. The third biopsy was secured immediately before initiation of second induction treatment. d=overall survival estimated as years from diagnosis/enrolment into study until death; c=event-free survival estimated as years from diagnosis/enrolment into study to disease progression/relapse. CR=complete response

Table 3 Genes altered more than 1.5 fold (P < 0.05) in the buccal mucosa of AML patients

Gene symbol	FC	p-value	adj. p-value	Qualified GO term	Function
Baseline versus healthy controls					
RNU6-996P	2,04	2,28E-07	0,0067	RNA, U6 Small Nuclear 996, Pseudogene	unknown
LINC01975	1.66	2.33e-06	0.030	Long Intergenic Non-Protein Coding RNA 1975	unknown
Day 2 versus baseline					
HIST1H1A	-3.20	8.08e-10	3.18e-05	Histone Cluster 1, H1a	transcription
HIST1H2BM	-2.83	2.48e-06	0.024	Histone Cluster 1, H2BM	transcription
POLH	2.18	1.97e-06	0.024	Polymerase; DNA directed	transcription
NOTCH1	1.85	2.04e-06	0.024	NOTCH 1	cell signalling

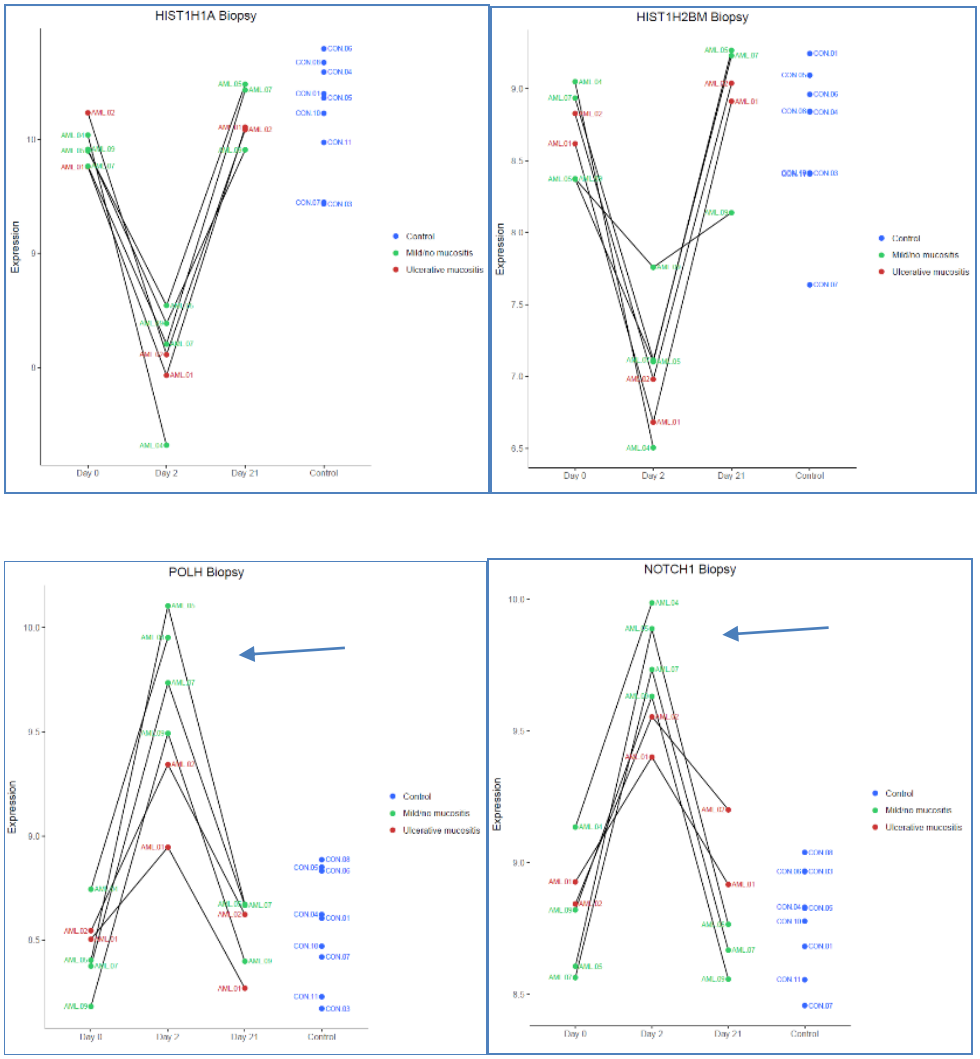
FIGURES

Figure 1. A detailed illustration of the study design.



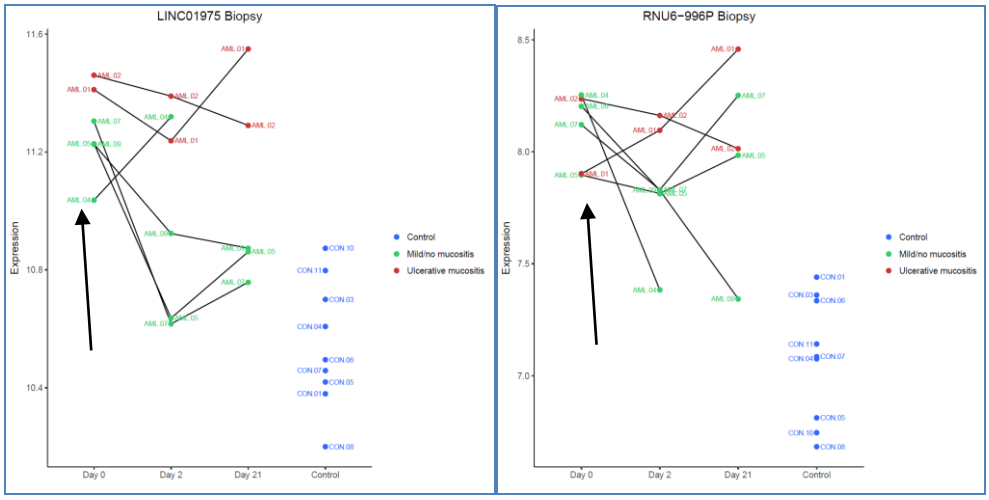
The 1st biopsy was collected before initiating induction therapy (Day0); the 2nd after 3-5 days of chemotherapy (Day3); and the 3rd biopsy was harvested at an out-patient control visit 19-41 day after the last chemotherapy session (Day27), just before initiating the second induction treatment. Tissues were stored successively in our biobank and subsequently analysed collectively in our laboratory.

Figure 2 Gene expression in the mucosa of AML during ADE induction treatment.



Gene expression in the mucosa of patients with AML receiving ADE induction treatment. before (Day0), after 3-4 days of chemotherapy (Day3), and before initiation of second induction therapy (Day27). Red = UM; green = NM; blue = CON. First row: genes of the histone family: *HIST1H1A* and *HIST1H2BMB*. Second row: *POLH* and *NOTCH1*. Although not statistically significant, there was a tendency towards upregulation of POLH and NOTCH1 in response to treatment among the patients that did not develop mucositis, see arrows. These genes were also differently expressed in the MM and TSCC group, but not with the same distinction.

Figure 3 Gene expression in the mucosa of patients with AML before treatment.

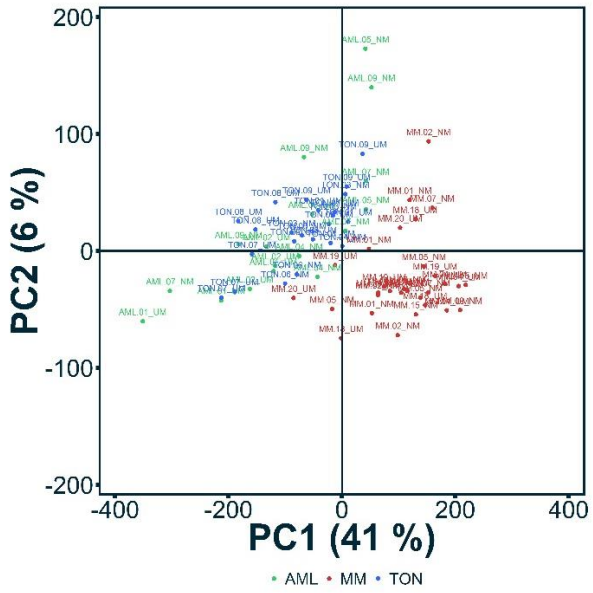


Gene expression of *LINC01975* (left) and *RNU6-996P* (right) before (Day0), after 3-4 days of chemotherapy (Day3), and before initiation of the second induction therapy (Day27). Red = UM; green = NM; blue = CON. The level of expression seems to normalize in response to treatment in some patients, see arrows.

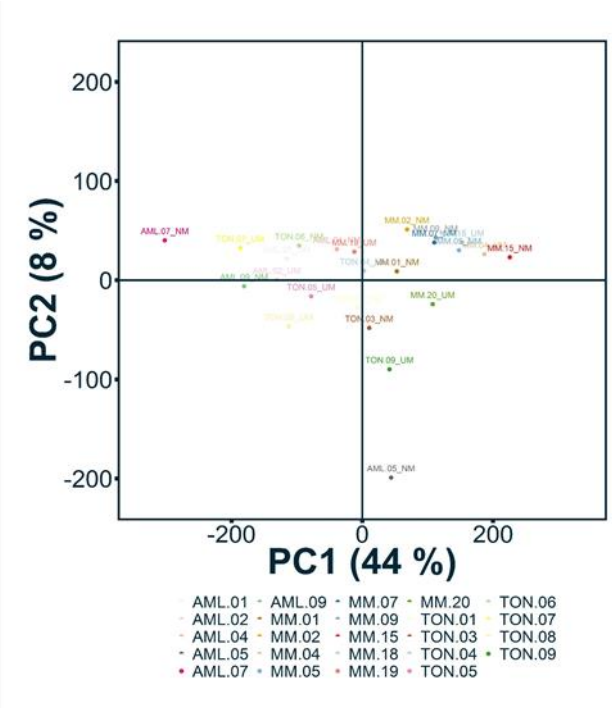
Figure 4 A and B. Principal components analysis of MM, TSCC, and AML

Three cohorts of patients with multiple myeloma (MM) = red (receiving high dose melphalan); AML = green (receiving ADE induction treatment); and tonsil squamous cell cancer (TSCC) = blue (receiving radiation treatment). A) Each patient delivered three consecutive biopsies before, during and after therapy. B) Only data from the 1st pre-treatment biopsies. Patients cluster according to disease indicating that disease is foremost contributor to the variation in gene expression of the mucosa samples and not mucositis grade.

A)



B)



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