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ORAL CHRONIC GRAFT-VERSUS-HOST DISEASE: CLINICAL AND PATHOLOGICAL STAGING

VICTOR TOLLEMAR

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Cover illustration: Schematic illustration of the healthy oral mucosal with progression of histopathological changes following haemopoietic cell transplantation and severe oral mucosal cGVHD. Illustrator Mats Ceder, Koboltart.

ORAL CHRONIC GRAFT-VERSUS-HOST DISEASE: CLINICAL AND PATHOLOGICAL STAGING
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Victor Tollemar

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Principal Supervisor:
Docent Rachael Sugars
Karolinska Institutet
Department of Dental Medicine
Division of Oral Diagnostic & Rehabilitation

Co-supervisor(s):
Assistant Professor Karin Garming-Legert
Karolinska Institutet
Department of Dental Medicine
Division of Oral Diagnostic & Rehabilitation

Professor Katarina Le Blanc
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Immunology & Transfusion Medicine

Professor Gunnar Warfvinge
Malmö University
Faculty of Odontology
Department of Oral Pathology

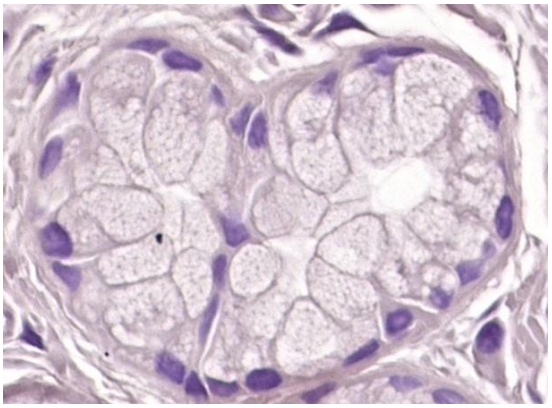
Opponent:
Professor Keith Hunter
University of Liverpool
Institute of Systems, Molecular and Integrative Biology
Molecular and Clinical Cancer Medicine

Examination Board:
Professor Karin Nylander
Umeå University
Department of Medical Biosciences
Division of Pathology

Docent Inger Wårdh
Karolinska Institutet
Department of Dental Medicine
Division of Oral Diagnostic & Rehabilitation

Assistant Professor Mattias Carlsten
Karolinska Institutet
Department of Medicine
Division of Hematology and Regenerative Medicine

To all allogenic HCT patients that currently and historically have been involved in medical research, and at the same time showing tremendous courage to fight their disease. All along, you have been the inspiration leading the work in this thesis.



To my beloved family, the interpretation of histopathology as with the daily life, allows you to find beauty within every situation. Haematoxylin-Eosin stained minor salivary gland acini. Acquired by Victor Tollemar.

POPULAR SCIENCE SUMMARY OF THE THESIS

Immune cells are important soldiers to protect against threats; commonly bacteria, viruses, and fungi. Some threats, as with various cancers, have developed properties to escape the immune soldiers and often result in poor outcome. Immune cells could also be wrongly programmed and hence attack the own body, leading to autoimmunity, which is hard to treat.

Immune and blood cells are created within the bone marrow and some individuals develop immune errors or cancers within this organ. In the past, these patients were associated with premature death. However, healthy donor immune cells can be given to patients through a bone marrow transplantation, to restore errors and defeat cancers. A major complication to overcome is the donor cell-related attack on the patient, called Graft-versus-Host disease (GVHD).

This thesis aims to increase our understanding of GVHD, a battle between donor immune soldiers (i.e. the graft) that target the patient (i.e. the host). Oral chronic GVHD (cGVHD) commonly occurs as a "autoimmune-like" attack with mouth soft tissue redness, sores, and scarring i.e. "Lichenoid-like reactions" or an oral dryness and discomfort due to dysfunctional salivary glands i.e. "Sjogren's syndrome-like". We investigated 303 oral tissue samples from 95 patients treated with bone marrow transplantation and 15 healthy volunteers.

Papers I and IV investigated the oral soft tissue and salivary gland pathology using microscopy. Oral soft tissue samples were reviewed, and disease patterns associated to cGVHD severity were found to be aggregates of immune cells, dead host cells and abnormal soft tissue and salivary gland structures. Donor cells were found to attack and destroy the oral tissue before onset of clinical symptoms, and that they persisted to cause damage even at late disease periods when the donor immune soldiers started to diminish. Classification of these disease reactions led to the establishment of pathological diagnostics.

We also performed experiments that identify and colour immune cells in the oral tissue samples i.e "Immunohistochemistry". **In paper II**, we compared an image analysis software (CellProfiler) with the traditional pathologist method to manually count identified immune cells in a microscope. We found a strong correlation between CellProfiler and the manual counting method. The benefits of computer aided analyses are timesaving, allows for standardisation and the possibility to have multiple measures performed.

In **papers III and IV**, we identified cGVHD immune cells in the oral soft tissue samples and measured these using CellProfiler. We found high levels of killer cells ("CD8"), which were associated to our pathological diagnostics, especially during the first 6 months of cGVHD. Helper immune cells ("CD4") were found at stable levels in the oral soft tissue and salivary glands. These can both trigger and control the activation of cGVHD but were mainly associated with mild and distinctive cGVHD, as well as at late stages of cGVHD. Immune cell cleaners ("CD68") were found in various clinical situations, from favouring a role in protection, to triggering damage, especially with persistent functions at late time phases of cGVHD. We also investigated cells responsible for antibody production ("CD19" and "CD20), and cells

responsible for presenting foreign substances (CD1a), but none of these were found to be dominant.

Oral cGVHD has been suggested to occur either in the soft tissue or the salivary gland, with only minor overlap. In **paper IV**, we found a strong association between oral pathology and immune cells, when cGVHD establishes, which suggests a systemic cGVHD activity. However, as cGVHD progresses, only a minor association was found, which implies that oral soft tissue and salivary gland cGVHD are two separate oral disorders.

Oral cGVHD is complex and our knowledge is limited due to research involving few patients. This thesis identified criteria for oral tissue sample evaluation and shed light on the role of various immune cells in different clinical severities, and time-points of oral cGVHD.

ABSTRACT

Allogenic hematopoietic cell transplantation (HCT) is a curative treatment for many patients with immune- hematopoietic disorders, mainly hematopoietic cancers as leukaemia. Chronic Graft-versus-Host Disease (cGVHD) is a major long-term complication, associated with mortality and morbidity following allogenic HCT. Oral cGVHD is common and might manifest as mucosal lichenoid manifestations (om-cGVHD) or with dysfunctional salivary glands (sg-cGVHD). Alloreactive T-cells respond to recipient tissues with pathological reactions of acute inflammation, progressing with chronic inflammation and dysregulated immunity, and subsequent aberrant fibrotic healing.

This thesis aimed to investigate diagnostic criteria for oral cGVHD using histopathological, clinical, and immune cell characterisation. A retrospective cohort of 95 HCT-patients and 303 oral biopsies were analysed, including 15 healthy controls. Oral mucosal biopsies with and without minor salivary glands (MSG) were retrieved from Stockholm Medical Biobank. Associated clinical information was gathered from the clinical charts and HCT register data. We applied histological (Haematoxylin and Eosin, Periodic acid Schiff, van Gieson), and immunohistochemical (IHC) staining (CD4 T-helper cells, CD8 T-cytotoxic cells, CD68 macrophages, CD1a Langerhans cells (LCs), CD19 and CD20 B-cells, and CD5 T-/B-cells). Quantitative IHC was performed using CellProfiler image analysis software.

In **papers I and IV**, oral mucosal-, and MSG histopathology were analysed in biopsies prior and post HCT, with and without cGVHD. We used the National Institutes of Health pathology criteria and formalised grading modules to assess pathology scores and grades (NIH cGVHD grading). The oral mucosa was observed with minimal criteria of lichenoid interface inflammation with exocytosis, liquefaction degeneration and apoptosis. Basal membrane alterations were the most specific criteria found. Features detected in the MSG were peri-ductal and acinar inflammation and exocytosis, destruction and fibrosis. We developed severity grades (G)0-IV and verified pathology diagnostics of "possible (GII)" and "likely (GIII-GIV)" cGVHD. In **paper IV**, we also employed the Greenspan composite MSG grading scheme, which was found with a strong correlation to the NIH cGVHD MSG grading.

IHC quantification was performed following established pipelines in CellProfiler, as described in **paper II**. The methodology was compared to manual counting, with a perfect concordance in detection of positive stained cells, as well as for positive stained regions. The benefit of CellProfiler is to perform standardised and repeatable quantification in a time saving manner.

Oral mucosal immune profiles were investigated in **paper III**. CD4 infiltration was associated with mild and distinctive om-cGVHD but were found with frequent stable levels over time. CD8 was elevated in clinical and pathologically severe om-cGVHD, particularly during cGVHD onset and progression. Immunolocalisation of CD68 revealed significant staining in various clinical groups, particularly at onset, but the association with severity was interesting especially during late stages of disease. CD1a LCs were significantly reduced in pathological

GII at onset and during progression, but otherwise non-significant compared to healthy. CD19 and CD20 were rarely observed.

In **paper IV**, we quantified the immune profiles in the MSG and found an altered pathology with significant increase of CD4- and CD8-cells. However, levels of B-cells and LCs were considerably low. The association between oral mucosal and MSG immunopathology, was investigated on the whole cohort and with respect to cGVHD duration. Overall, a moderate correlation was observed for pathology scores, CD4 and CD8 infiltrate. Interestingly, at the time of cGVHD onset, the correlation between the oral mucosal disease and MSG was stronger but with progression no further association was found.

In conclusion, om- and sg-cGVHD are two heterogenous complications that display associated immune-pathology profiles during cGVHD onset, but progression appears to be tissue-dependent. We developed histopathological grading modules to facilitate severity diagnostics, which were significantly associated with CD4, CD8 and CD68 immunostaining. om-cGVHD clinical and pathological characterisation was found associated to changes in the immune profile. CD8 was found to drive the severe disease reaction during onset and progression but diminished over time. However, long duration of the disease correlated with elevated CD68 and persistent CD4 cells. This highlights the need for improved clinical and pathological characterisation in combination with biological disease classification.

LIST OF SCIENTIFIC PAPERS

I. **Victor Tollemar**, Nikolce Tudzarovski, Gunnar Warfvinge, Noam Yarom, Mats Remberger, Robert Heymann, Karin Garming-Legert, Rachael Victoria Sugars. Histopathological Grading of Oral Mucosal Chronic Graft-versus-Host Disease: Large Cohort Analysis. Biol Blood Marrow Transplant. 2020 Oct;26(10):1971-1979. doi: 10.1016/j.bbmt.2020.06.031. Epub 2020 Jul 10. PMID: 32659433.

II. **Victor Tollemar***, Nikolce Tudzarovski*, Erik Boberg, Anton Andrèn Törnqvist, Amir Al-Adili, Katarina Le Blanc, Karin Garming-Legert, Matteo Bottai, Gunnar Warfvinge, Rachael Victoria Sugars. Quantitative Chromogenic Immunohistochemical Image Analysis in Cellprofiler Software. Cytometry A. 2018 Oct;93(10):1051-1059. doi: 10.1002/cyto.a.23575. Epub 2018 Aug 8. PMID: 30089197. *contributed equally

III. **Victor Tollemar**, Jennifer Ström, Nikolce Tudzarovski, Henrike Häbel, Karin Garming-Legert, Robert Heymann, Gunnar Warfvinge, Katarina Le Blanc, Rachael Victoria Sugars. Immunohistopathology of Oral Mucosal Chronic Graft-versus-Host Disease Severity and Duration. *Manuscript*

IV. **Victor Tollemar**, Arvidsson Helena, Henrike Häbel, Nikolce Tudzarovski, Karin Garming-Legert, Katarina Le Blanc, Gunnar Warfvinge, Rachael Victoria Sugars. Minor Salivary Gland Immuno-Histopathology in Chronic Graft-versus-Host Disease: Association with Oral Mucosal Disease and Duration. *Manuscript*

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LIST OF ABBREVIATIONS

APCs	Antigen presenting cells
BAFF	B-cell activation factor
BMSCs	Bone marrow stem cells
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
DAB	Diaminobenzidine
DCs	Dendritic cells
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
GVHD	Graft-versus-Host Disease
aGVHD	Acute GVHD
cGVHD	Chronic GVHD
om-cGVHD	Oral mucosal cGVHD
sg-cGVHD	Salivary gland cGVHD
GI	Gastrointestinal
gl.	Glandular
GVL	Graft-versus-Leukaemia
HCT	Hematopoietic cell transplantation
HE	Haematoxylin and Eosin
HLA	Human Leukocyte Antigen
IHC	Immunohistochemistry
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
KUH	Karolinska University Hospital
LCs	Langerhans cells
LR	Likelihood ratio
MAC	Myeloablative conditioning
MHC	Major histocompatibility complex
MSG	Minor salivary gland

NIH	National Institutes of Health
NK-cells	Natural Killer cells
OLP	Oral lichen planus
OMRS	Oral mucosal rating scale
OPMD	Oral potential malignant disorders
PAS	Periodic Acid Schiff
PBSCs	Peripheral blood stem cells
RIC	Reduced intensity conditioning
SMB	Stockholm Medical Biobank
SS	Sjogren's syndrome
T_c-cells	T-cytotoxic cells
T_h-cells	T-helper cells
T_{reg}-cells	T-regulatory cells
TNF	Tumour necrosis factor
vG	van Gieson
WSI	Whole slide imaging

1 INTRODUCTION

Oral Graft-versus-Host Disease (GVHD) is a complication following allogenic hematopoietic cell transplantation (HCT), a treatment for patients with immune hematopoietic disorders as leukaemia [1, 2]. The infused hematopoietic cells aim to repopulate and restart the patient's hematopoietic system. Transplanted cells could be autologous (stem cells from the recipient) or allogenic (stem cells from a donor), and are dependent on patient status, the underlying disease, as well as the overall risk [1, 2]. Within this thesis, we will focus on allogenic transplantation, referred to using the abbreviation HCT.

A major benefit of allogenic HCT is the ability of the infused donor cells to immunologically target any remaining cancer cells; the Graft-versus-Leukaemia (GVL) effect [2, 3]. However, the infused cells might also initiate GVHD, where immune competent donor cells respond to the host environment as foreign, leading to autoimmune-like inflammation, immune dysfunction and fibrosis [4, 5]. GVHD is the major non-relapse related complication after HCT, and often affects multiple organs and tissues [5, 6]. GVHD involves different pathophysiological pathways but is broadly described as acute (aGVHD) or chronic (cGVHD) [7]. aGVHD typically involves the skin, upper and lower gastrointestinal (GI) tract and the liver, whereas cGVHD commonly involves the skin, liver, mucosal surfaces and glands (mouth, genital and eye), but also other organs and sites [5, 7].

Oral complications after HCT are common and often related to increased morbidity and decreased quality of life [8]. Acute oral complications, such as mucositis, bacterial, Candida and viral infections are primarily associated to the conditioning (chemotherapy with or without irradiation) given before HCT or to the high-dose immunosuppression post-HCT [9-13]. Patients further complain of xerostomia, taste dysfunction and general pain, which interferes with their social life and food intake [8, 14-16]. Many of these issues and symptoms follow long-term, with additional manifestations of oral cGVHD. As one of the primary and earliest organs affected, cGVHD manifests in the oral cavity with mucosal lesions, salivary gland dysfunction and restricted mouth opening [15, 17, 18]. Over time, these patients have increased risk for caries and periodontitis, as well as an altered risk for secondary oral malignancies [11, 15, 19, 20].

Oral cGVHD has been well recognized since the first use of HCT [21, 22]. However, the field has been hindered by research involving small cohorts with a lack of clear patient descriptions. In recent years, our understanding of the pathological conditions involving mucosal and glandular tissues has evolved [18]. The need for better diagnostic and phenotypic criteria is urgent. Therefore, the aim of this thesis focuses on oral cGVHD, to increase our understanding of the pathological and immunological profiles underlying the different clinical symptoms and phases.

2 ALLOGENIC HEMATOPOIETIC CELL TRANSPLANTATION

2.1 A BRIEF HISTORICAL OVERVIEW

The idea behind HCT evolved from radiation studies in World War II atomic bomb survivors in Japan. Stem cell injections into irradiated mice were seen to populate the bone marrow, which gave rise to a potential clinical intervention against irradiation. In 1957, Thomas and colleagues published their report of the first end-stage leukaemia patient treated with an HCT-like infusion [23]. However, the enthusiasm for HCT trials diminished during the following decade since none of the HCT patients survived. Graft failure, infections, relapse and GVHD were necessary obstacles to overcome for the clinical benefit of HCT. A breakthrough occurred in the 1970s with the understanding of matching donor and recipient cells to avoid graft failure and GVHD [24]. The 1990 Nobel Prize in Medicine and Physiology was awarded to Edward Donnall Thomas for his research discoveries concerning cell transplantation. Today, more than 1.5 million of autologous and allogenic HCTs have been performed, with an annual increase of more than 80 thousand [25, 26]. Karolinska University Hospital (KUH), Center for Cell Therapy and Allogeneic Stem Cell Transplantation, has demonstrated an improved overall survival rate with the development of HCT standards [27, 28]. However, HCT places an extreme burden on national health care systems, where HCT contributes to enormous and increasing costs, with treatment strategies and complications that result within the first year [6, 29]. Following increased numbers of HCTs worldwide, the long-term survival continues to be improved. However, as a consequence, cGVHD prevalence is also increasing in demand with the need for improved patient management [6, 30].

2.2 INDICATIONS FOR HCT

Indications for HCT include immunodeficiency, hemoglobinopathies, severe autoimmune disorders and bone marrow failure, such as Fanconi anaemia and aplastic anaemia, which are all non-malignant immunohematopoietic conditions [1]. Malignant disorders within the lymphoid cell-line that may be suitable for immunotherapy, include acute lymphoblastic leukaemia, chronic lymphoblastic leukaemia, myelomas, and various lymphomas including B-cells, non-Hodgkin`s, Hodgkin`s and follicular [31]. Malignant transformation in the myeloid cell-line can cause acute myeloid leukaemia, chronic myeloid leukaemia and other myeloproliferative diseases, such as myelofibrosis [31]. HCT is a pioneering treatment and globally the most used approach within the field of cellular immunotherapy [2]. Different immunotherapy approaches are constantly developing with clinical trials conducted worldwide [32]. Pharmaceutical approaches, such as the development of tyrosine-kinase inhibitors used for chronic leukaemia, as well as intense chemotherapy in acute lymphoblastic leukaemia, reduced the need for HCT as a first-line option [31]. However, in many cases HCT remains the only curative treatment, especially with respect to high-risk hematopoietic cancers, diminished treatment outcomes, as well as in refractory and relapsed patients [31, 33].

2.3 HCT PROCEDURES

Rigorous examinations and procedures prior to HCT involve medical assessment for comorbidities and eligibility for HCT. Dental examinations are also required to confirm infection-free status and lower the risk of local trauma [9, 12, 34, 35]. HCT procedures include stem cell harvest and pre-treatment (conditioning) before the actual cell infusion. Subsequently, patients continue with clinical observations post-HCT, to follow immune reconstitution, with tapering of any immunosuppressive treatment and management of any transplant-related complications.

2.3.1 Selection of donor cells

Discovery of the human leukocyte antigen (HLA) complex led to the ability to match patient and donor immunity [24]. The HLA corresponds to major histocompatibility complex (MHC) types I and II, which is key in immunological responses and in transplantation immunology. Every nucleated cell expresses class I, whereas class II is presented on antigen presenting cells (APCs), like dendritic cells (DCs), macrophages and B-cells [24]. Selection of a suitable donor has evolved with the many international bone marrow donor registries. However, most registered donors are Caucasian (~70%) and as a result it is harder to find suitable donors for recipients of other ethnicities [36]. The first registry was established in London 1974, founded by the family members of a 3-year-old boy who needed HCT, the Anthony Nolan Trust. In 1991, Sweden's national Tobiasregister (www.tobiasregistret.se) was established, and currently has more than 200,000 donors listed. The search for a donor often occurs within the international register (wmda.info), which currently lists more than 39 million potential donors.

A good HLA match of recipient to donor minimises the risk of donor T-cells responding to recipient self-antigens [24]. However, HLAs are highly polymorphic with enormous different alleles on HLA-I genes (-A, -B and -C,) and HLA-II genes (-DR, -DQ and –DP) [37]. Suitable donors might be any HLA-matched relative or HLA-matched unrelated donor identified within registers [37]. In addition to the HLA-match, the recipient and donor are also screened based on gender, age and serology for cytomegalovirus (CMV) [36, 38]. Despite the well-established donor registers, it can be hard to find a perfect match but developments in transplantation technologies have allowed for the possibility of using more accessible haploidentical relatives that requires matching of only one of two HLA haplotypes [39].

2.3.2 Conditioning

Conditioning prior to HCT has been motivated due to its ability to affect the underlying disease processes by targeting cancer cells and suppressing the immune system in preparation for new stem cell engraftment [2]. Strategies for myelosuppressive conditioning varies between using combinations of chemotherapy and/or irradiation. Conventional HCT involved bone marrow stem cells (BMSCs) and myeloablative conditioning (MAC), i.e. high-intensity total body irradiation (TBI) and high-dose chemotherapy [40]. MAC lowers the risk of relapse, but pancytopenia and toxicity increase the risk for HCT-related mortality [40]. The shift to modern HCT techniques included reduced-intensity preparative conditioning (RIC), and during the

1990`s, the introduction of peripheral blood stem cells (PBSCs) as an alternate graft source [40]. This enabled older impaired patients to be enrolled into HCT and results in less cytopenia [2].

2.3.3 Stem cell infusion

Allogenic donor cells can be retrieved from BMSCs, PBSCs or the umbilical cord. These sources of stem cells have different properties, for example PBSCs more rapidly engraft but also gives rise to higher GVHD/GVL due to high levels of T-cells in the graft [40]. Therefore, harvest from bone marrow could be more beneficial, especially in younger patients at early disease stages or with patients with non-malignancies, like multiple sclerosis, that do not require the GVL effect. In scenarios when HCT is urgent or without HLA-identical match, stem cells can be quickly harvested from the umbilical cord, which are not as sensitive for HLA mismatch in the context of GVHD risk [31, 40]. Whichever type of stem cells are selected, transplantation occurs through infusion using a central venous catheter and consists of a heterogenous population of hematopoietic stem cells, progenitors and mature cells [41].

2.4 IMMUNITY AND RECONSTITUTION

The immune system is a complex interaction of cells, signals, and tissues and is crucial for human survival. Immunity consists of innate and adaptive components, that protect from pathogens, and defeat disorders, such as cancers, by distinguishing self and non-self. Undesirable reactions of the immune system may involve autoimmunity and allergies. The patient`s immunity will be depleted following HCT regimen. Therefore, engraftment of donor cells is vital for immune reconstitution. Restoration of the T- and B-cell repertoire is desired for long-term protection from infective microorganisms, but also short-term for GVL [4]. However, in cases of alloreactive reconstitution, GVHD will initiate due to donor cells targeting recipient tissues [4]. Introduction of GVHD prophylaxis, using the folic acid antagonist, Methotrexate and the calcineurin inhibitor (CNI) Cyclosporine, reduced the lethal events of aGVHD [29]. Immunosuppressive agents routinely used today to ameliorate GVHD include combination of T-cell depletion, short course of chemotherapy, i.e., Methotrexate, Cyclophosphamide, and CNIs, like Cyclosporine, Tacrolimus or Sirolimus [42, 43]. Although, drawbacks to such prophylaxis result in delayed immune reconstitution and increased risks of infections [44]. Consequently, patients require re-vaccination and prophylaxis agents against bacterial, viral and fungal infections. The following sections briefly reflect upon normal innate and adaptive immunity with the reconstitution post-HCT.

2.4.1 Innate immunity

Innate immunity is a rapid and relatively non-specific reaction to threats. The skin and mucosal barriers are the first line of defence, but the complement system and antimicrobial peptides are also immunologically important. Innate immune cells respond when barrier integrity is lost and include macrophages (referred to as CD68 within this thesis), DCs (referred to as CD1a within this thesis), neutrophils, natural killer (NK) cells, and innate lymphoid cells [45]. Following the

neutropenic phase after pre-HCT conditioning and ongoing immunosuppressive therapy, patients are susceptible for acute infections [46-48]. The oral mucosal barrier is commonly affected by conditioning, and saliva functions are impaired [9]. Large numbers of neutrophils are restored within the first month post-HCT, abating the risk for severe acute infections [45, 48]. Recipient macrophages are believed to be more resistant to conditioning, so that resident host macrophages will persist over many months [49]. The recipient/donor chimerism of skin and mucosa resident DCs, Langerhans cells (LCs), might depend on conditioning regime but irrespective of conditioning LC levels are thought to be depleted [49-52]. Patients with aGVHD have been found with a slower recovery of the LC population up until the first year [51]. The chimerism of host/donor macrophages and LC populations remain to be understood in terms of cGVHD.

2.4.2 Adaptive immunity

Adaptive immunity is more specialised, but slower compared to the innate immune response. Antigen recognition links innate and adaptive immune response through professional innate APCs, i.e. macrophages and DCs. Engulfed foreign antigens are presented on MHC class II receptor to T-helper (T_h) (CD4) cells. T-cytotoxic (T_c) (CD8) cells respond to MHC class I presentation either by cells with an intra-cellular infection/process or through APC MHC class I cross-presentation. Concurrently, both innate cells; as macrophages and NK-cells, and adaptive cells; as T-cells, secrete cytokines and chemokines for co-stimulation and recruitment of immune cells, such as interferon (IFN)γ, tumour necrosis factor (TNF)α, interleukin (IL)-2, and IL-6 [7, 44, 53]. Central tolerance is impaired as the thymus could be damaged by acute and cGVHD, leading to a lack of negative selection and continued alloimmunity in GVHD patients [44]. As such, the main recovery of T_c-cells and T_h-cells, in adults occurs after 3-6 months, but these are thymic-independent naïve memory T-cells that are either resident host cells or infused donor cells [44, 45]. Thymic-dependent maturation of new T-cells first occurs into the second year after HCT [44]. However, it should be noted that absence of both early effector and late naïve T_c-cells have been noted in some patient reports [44]. B-cells (referred to as CD19 and CD20 within this thesis), representing the humoral immunity, are professional APCs with the capacity to further recognize specific antigens through their immunoglobulin (Ig)-receptors. Upon recognition, B-cells produce circulating Ig that activates the complement system and binds to pathogens to trigger phagocytosis or for blocking signals. Reconstitution of B-cells is slow, with immature B-cells circulating after a few months post-HCT, but full recovery and function may not be restored until 1-2 years, which is further delayed in cGVHD [45, 48].

3 GRAFT-VERSUS-HOST DISEASE

GVHD was first described as a complication post-HCT by Billingham in 1966 [54]. Billingham described GVHD to occur due to immunocompetent donor immune cells responding to an immunologically different recipient, and because of immunosuppression, the host lacked the regulatory functions to halt the alloreactive GVHD response [54]. GVHD manifests as two classic forms: aGVHD and cGVHD. Traditionally, aGVHD has been defined as symptoms occurring within the first 100 days after HCT [55, 56]. In contrast, cGVHD symptoms manifested post 100 days after HCT, and for most patients within the first year [56, 57].

3.1 DEFINITIONS

The field of cGVHD has been steered following the National Institutes of Health (NIH) Consensus Development Projects in 2005 and 2014, focusing on recommendations for diagnosis and staging to improve the outcome of clinical trials [5, 6, 55, 58-63]. The staging of GVHD based on time has been reformulated, highlighting a deeper understanding of the differences between aGVHD and cGVHD clinical status and symptoms [5, 55]. Different types of aGVHD have been established and included, in addition to the classic aGVHD, persistent late aGVHD continuing >100 days, recurrent late aGVHD with resolution but new onset >100 days, and de novo aGVHD that initiates >100 days without previous signs of aGVHD [56]. To submit that cGVHD is a resumption of previous aGVHD ("quiescent" cGVHD onset) is not truly correct [64]. cGVHD could onset without previous aGVHD ("de novo" cGVHD onset), whereas continuation of acute symptoms into cGVHD is classified as "progressive" cGVHD onset [56]. The NIH Consensus Development Projects suggested that cGVHD is classified as either a stage of overlap, when concurrent acute and chronic disease signs present simultaneously or classic cGVHD when no lesions or signs of aGVHD are present [55, 56]. Recently, the NIH working groups followed up with a third Consensus Development Project published in 2020. These documents focused on the basic and clinical needs for improvement, including aetiology/prevention, diagnosis/pre-emptive therapy, systemic treatment, and highly morbid forms of cGVHD [6, 65-68].

3.2 ACUTE GVHD

The classical and specific manifestations of aGVHD are typically skin, liver and GI tract involvement [21, 56, 69]. Clinical staging measures the amount of erythematous skin rashes, bilirubin levels and diarrhoea [56]. Other organs might be involved but few if any oral complications have been reported [70]. aGVHD is the major short-term risk for mortality and therefore accurate diagnostic tests might be required to rule out other differential conditions [71]. Biopsies display heavy inflammatory infiltrate that targets the epithelium with signs of apoptosis [7]. The severity classification (Grades 1-4) was described in the 1970s and remains in clinical practice today, with Grades 3-4 associated with poor survival outcome [72]. Beside the GVHD prophylaxis of CNIs, other T-cell depletion regimes have been tested to ameliorate the

clinical situation, such as anti-thymocyte globulin [73, 74]. However, many of these approaches followed with an increased risk of infections and relapse [42, 73, 75]. Treatment for aGVHD includes high-dose systemic or topical corticosteroids.

3.3 GRAFT-VERSUS-LEUKEMIA (GVL) EFFECT

Graft-versus-Host reaction includes GVHD, but also the alloreactive GVL response targeting malignant cells. Thus, the clinical impediment of cGVHD is balanced with the protective role against cancer relapse. Patients with GVHD, especially those with cGVHD, show less occurrence of malignant relapse [75]. This concept is used clinically, with quick tapering and discontinuation of immunosuppressants in patients needing a Graft-versus-Host reaction. Furthermore, post-HCT relapse is treated using donor lymphocyte infusion [76]. T-cell and NK-cell reactivity is fundamental for GVL; however, it remains to be explored whether CD4 or CD8 T-cells are the main component in GVL [76]. TNFα is important for GVL but also association to GVHD development [77, 78]. Regulatory T-cells (T_{reg}-cells) are most likely the key to limit and supress GVHD but preserving the GVL effect [79]. Of note, evidence also points out a beneficial role for NK-cells with improved transplant engraftment and decreased GVHD due to elimination of recipient cells involved in the different pathways [76, 80].

3.4 CHRONIC GVHD

Active manifestations of cGVHD impact patients' quality of life with decreased general and mental health, impaired functionality and activity, and elevated pain and anxiety [81]. The impact of such disabilities has been shown in patients with isolated oral-, oral and extra oral- and non-oral cGVHD [8]. Risks associated with GVHD establishment have differed between reports, suggesting donor sex, age and match, stem cell source, conditioning regime, underlying disease, prior CMV/Epstein Barr virus infections and post-HCT antibody T-cell depletion, as well as Cyclophosphamide [55, 82-84]. Following the NIH cGVHD diagnostic criteria from 2005, a large single-centre study of almost 3,000 HCT-patients found prior aGVHD, as well as the following risk factors (independent of aGVHD) for cGVHD: mismatched or unrelated donor, elderly donor and patient, female donor to male recipient and PBSCs [64].

3.4.1 Manifestations and diagnosis

In general, diagnostic cGVHD commonly involves the skin, mouth, GI tract, lung, genital, and fascia, but distinctive sites as the eyes and liver are also common [56]. The first classification of cGVHD was described as limited or extensive, focused on skin and liver presentation [21]. Today, lung involvement is considered with a high disease burden, but other highly morbid forms include advanced skin and fascia sclerosis, ocular and GI involvement [5, 85]. There is also an unmet need to define morbid forms of oral cGVHD that impact quality of life with increased risk of secondary cancer [85]. In general, approximately 30%-50% of patients surviving HCT develop cGVHD, most within the first year but delayed onset is possible [56].

Current guidelines are based on the NIH Consensus global severity scoring system; score 0: no GVHD, score 1: mild GVHD, score 2: moderate GVHD, and score 3: severe GVHD. The global scoring involves an eight-item form assessing the skin, mouth, eyes, GI tract, liver, lung, joints and fascia, and genital tract. Additional performance scores are assessed but not incorporated into the severity score [5]. The NIH organ specific score for cGVHD mouth involvement is focused on mucosal disease manifestations [5]. Oral cGVHD severity diagnostic score (0-3) does not include type or distribution of lesions but is rather defined by symptoms and limitation of oral intake, ranging from none to severe (Table 1) [5]. The systemic organ evaluation of disease status results, in the overall global severity score (0-3) (Table 1).

Table 1. The National Institute of Health Consensus Development Project on cGVHD Scoring Criteria. A four-point scale (score 0-3) was defined for organ specific severity and a final cGVHD global diagnosis. Assessment for oral clinical disease combines a range of symptoms and limitations on oral intake. The overall cGVHD severity is based on the number of organs scored with 0-3 but with the lung score weighted separately. *Adapted from NIH Consensus Development Project Diagnosis and Staging working group* [5].

	SCORE 0	**SCORE 1**	**SCORE 2**	**SCORE 3**
Oral cGVHD organ scoring	No symptoms	Mild symptoms with disease signs but not limiting oral intake significantly	Moderate symptoms with disease signs with partial limitation of oral intake	Severe symptoms with disease signs on examination with major limitation of oral intake
Global severity of cGVHD	No cGVHD – – – – – Organs with score 0	1-2 organs with score 1 + Lung score of 0	≥ 3 organs with score 1 – – – – – – – ≥ 1 organs with score 2 – – – – – – – Lung score of 1	≥ 1 organs with score 3 – – – – – – – Lung score of 2 or 3

The NIH also presented cGVHD therapeutic outcome measurements [60]. To assess treatment responses and evaluate disease activity, patient and clinician assessment tools were recommended. These included clinical measurements of disease manifestations and symptoms, as well as patient reported issues. For the oral cavity a modified oral mucosal rating scale (OMRS) was suggested, as well as mouth sensitivity scale, where irritation resulting from normally tolerated spices, foods, liquids or flavours were measured as an outcome for cGVHD activity [60]. The OMRS tool, which was initially developed in 1992, was designed to objectively assess and diagnose the pattern and extent of clinical mucosal lesions [86]. This model was later adapted, from the original NIH scoring of lesions 0-15 in 2005 [63], to the modification (scoring lesions 0-12) in 2014 [60], where the assessment of mucoceles was removed [60, 87, 88]. Prior to the 2014 NIH guidelines, reports suggested scores ≥3 to be assigned as oral cGVHD and scores of 0-2 to be inconclusive for oral cGVHD [89] Furthermore, clinical improvement or worsening of <3 could be due to inter-rater variability [90].

3.4.2 Biology

GVHD pathobiology is complex but since being originally described by Billingham, our knowledge has advanced considerably [4]. The immunocompetent cells are well-known to be T-cells, which respond to the genetically different HLAs [7]. Over the years a three-step model has evolved describing GVHD biology [4]. The first phase involves inflammatory components associated with tissue damage due to the given conditioning treatment. Phase two occurs with the activation of adaptive effector immune cells in the presence of dysregulated immunity. Continuing disease propagation and aberrant tissue repair might involve increased pro-fibrotic mediators leading to organ dysfunction in phase three [4].

The model originates from the GI tract mucosa, where the mucosal barrier is disrupted due to chemotherapy-associated mucositis [7]. In phase one, acute inflammation is triggered by leakage of pathogen-associated molecular patterns, as lipopolysaccharides. Host tissue damaged-associated molecular patterns is realised, including proinflammatory cytokines; TNFα, ILs-1, -6 and -12 [4, 53]. Innate immune cells, such as macrophages and DCs are activated through their Toll-like receptors and migrate to lymph nodes, leading to enhanced antigen presentation and T-cell differentiation [4]. An acute immunity cascade initiates with the activation of naïve T_h-cells, with polarisation and expansion into T_h1- and T_h17-cells, secreting the cytokines IFNγ, IL-2, IL-17 and IL-22 respectively [7, 53]. The paradigm of T_h1-/T_h2-cell involvement has been discussed in terms of acute/early and chronic/late GVHD pathogenesis, but without consistent data supporting either pathway [4, 91].

Chronic inflammation with increased IFN levels, recruit effector T_h1/T_c1 cells into the target tissue and amplifies cGVHD in phase two. A potential protective role for this process includes IFNγ-induced T-cell apoptosis [4, 92]. IFNγ further stimulates the production of homeostatic B-cell activation factor (BAFF), the frequency of which has been increased in patients with GVHD [4, 93]. Elevated BAFF levels are associated with delayed B-cell reconstitution but also increased B-cell receptor signalling and cGVHD severity [93-95]. B-cell biology with associated auto- and alloantibodies has gained interest over the past decade [96]. T_c-cells are the main

effectors of cGVHD but the coordinated T_h-cell, B-cell and macrophage response, with a cytokine cascade, and production of antibodies, remains to be fully understood [96, 97]. T_{reg}-cells (FoxP3/CD4+CD25+) have an important function to suppress and control the alloreactive response [4]. To add to the complexity, IL-2 activate T-cell differentiation and expansion, as well as generating and maintaining T_{reg}-cells [7]. The IL-2 receptor is also target for the widely used CNIs [7].

Chronic inflammation often results in impaired would healing, abnormal tissue architecture and dysfunctional fibrosis [4]. Phase three is characterised by the activation of extracellular matrix (ECM) components, typically due to differentiated myofibroblasts leading to the pathogenic stages of fibrosis [4, 98]. Transforming growth factor β is a hallmark cytokine for the initiation of profibrotic processes, secreted by many cell types including tissue macrophages, however immune components responsible for sustained fibrosis are not well known [4, 99]. Differentiated B-cells, plasma cells with Ig deposition, as well as T_h2-, T_h17- and T_{regs}-cells are also known to be involved in the profibrotic stages but this likely to be organ-dependent pathways [4, 99]. To overcome cGVHD, in theory alloreactive donor T-cells should be depleted, T_{reg}-cells and thymus function need to be restored, and tissue repair and fibrosis may stop the progressive GVHD reaction [4].

3.4.3 Treatment

Management of cGVHD is based on clinical severity and organ dysfunction. Mild cGVHD are first treated with topical steroids or CNI agents, and systemic corticosteroids are used for patients with moderate to severe cGVHD. As such, first line treatments often include a combination of Prednisone with or without CNIs, but as many as 50% of cGVHD patients become steroid refractory and demand a second line treatment within the first two years post-HCT [100, 101]. Increased understanding of the different pathophysiologies involved in cGVHD, has led to multiple trials focused on investigating therapeutics related to specific cGVHD pathways rather than using broad immunosuppressants [68].

Many options are available for second line treatments, but no consensus or patient-steered recommendations are available for steroid refractory disease [6]. Therapies might involve extracorporeal photopheresis, B-cell depletion (Rituximab), anti-metabolite immunosuppressant (Mycophenolate Mofetil), chemotherapy (Methotrexate), and many other biological drugs are currently being investigated in clinical trials (reviewed in Saidu et al., and Wolff et al) [42, 100-103]. In recent years, three drugs were approved by the United States Food and Drug Administration [104]. All three (Ibrutinib, Ruxolitinib, Belumosudil) belong to the family of kinase inhibitors and are authorized as second- or third-line treatments for cGVHD [104]. Of note, Ruxolitinib has shown a good response in a cohort of 53 steroid refractory oral cGVHD patients [105].

4 THE ORAL CAVITY

4.1 ORAL MUCOSA HISTOLOGY AND PHYSIOLOGY

The oral mucous membrane is part of the body's outer barrier to protect against trauma, microorganism, and toxicity. The mucosa further involves receptors for perception of temperature, pain, sensation, and taste. The histological structure consists of an oral stratified squamous epithelium and underlying lamina propria of loose connective tissue. The oral mucosa epithelium is organised with distinct stratified cell layers with the superficial layer being either keratinised, as seen within the tightly attached masticatory mucosa of the palate and gingiva, or non-keratinised as with the flexible lining mucosa, such as the buccal mucosa and floor of the mouth. The dorsal tongue mucosa is a specialised structure with papillae and taste buds.

Oral keratinocytes are attached to each other through desmosomes, while the basal membrane, a thin protein-polysaccharide structure, separates the basal cell layer of the epithelium and lamina propria through hemidesmosomes. Other cells, such as Merkel cells, LCs, melanocytes and inflammatory cells, are also located in between the keratinocytes. Epithelial renewal and turn-over starts from proliferating basal cells, and over a 24-day period differentiation through the epithelial layers occurs [106].

The keratinocyte derived basal lamina constitute a part of the basal membrane, properly described in terms of lamina ludica and lamina densa. The basal membrane consists of ECM proteins, including laminins and collagen type IV [107]. The underlying lamina propria is rich in the ECM proteins, collagen, mostly types I and III, and elastin fibres, and forms the papillary and reticular supporting network. The abundant cell type is fibroblasts, which synthesise the matrix components, including collagens III and IV of the basal membrane lamina fibroreticularis, attaching into the basal lamina. Acute and chronic inflammatory cells, such as granulocytes and lymphocytes are also commonly observed in the lamina propria. Deeper into the oral mucosa membrane is a submucosal structure with tighter connective tissue filaments, adipose tissue, and minor salivary glands (MSGs).

4.2 SALIVARY GLAND HISTOLOGY AND PHYSIOLOGY

Salivary secretion is important not only to lubricate the oral mucosa and maintain homeostasis for the oral milieu but for maintenance of tooth integrity, antimicrobial effects, and functions related to taste, mastication, and speech [108]. We have three paired major salivary glands, glandular (gl.) parotis, gl. submandibularis and gl. sublingualis, which are located outside of the oral cavity with longer ducts leading saliva secretion to the oral cavity. However, around 10% of secreted saliva is created within the MSG, located in the submucosa and, most commonly, in the labial mucosa and soft palate.

The exocrine salivary glands have a secretory unit of specialised epithelial acinar cells, and small intercalated ducts, larger striated ducts and excretory ducts. Acinar units and their closely associated ducts are of cylindric or cubical epithelial cells. Whereas as closer to the mucosal membrane the ducts display stratified epithelial structures. The acini and ducts are structured as lobules, often referred to as the functional parenchyma and surrounded by loose connective tissue. Larger salivary gland lobes are separated with connective tissue septa.

Cells in the parenchyma are mainly fibroblasts, plasma cells and acinar-contractile myoepithelial cells. Primary saliva is created by acinar units and to some degree even by the intercalated duct cells. The latter also modifies the saliva with secretion of molecules including lysozyme and lactoferrin. As the saliva flows throughout the ductal system a process of re-absorption and outflow of electrolytes modify the saliva. Resulting saliva is either watery (serous) or viscous (mucous). Different salivary glands are often referred to as being either serous or mucous, with gl. parotis 100% serous, gl. submandibularis 80% serous, gl. sublingualis 50% serous. Most MSGs are mucous producing.

5 ORAL GRAFT-VERSUS-HOST DISEASE

5.1 CLINICAL AND HISTOPATHOLOGICAL CRITERIA

Manifestations of oral cGVHD resemble other autoimmune syndromes within the oral cavity. Oral lichen planus (OLP) and oral mucosal (om-)cGVHD affect the mucosal surfaces with typical white striations, erythema and ulcerations, whereas Sjogren's Syndrome (SS) and salivary gland (sg-)cGVHD display sicca syndrome-like symptoms and mucoceles. Historically oral cGVHD has often been reported as one consolidated disorder. As such, the two modules for oral cGVHD histopathological grading, published in 1981 and 1995 respectively, combined salivary gland and oral mucosal severity feature criteria [22, 109]. The NIH cGVHD Consensus Pathology Working Group in 2005 designed a histopathology consultation form, which included features for evaluation of MSG and oral mucosal pathology [61].

The prevalence and description of cGVHD remains to be improved. However, oral cGVHD is recognised as the first visible site, and one of the most frequently affected organs after HCT (45-83%) using both BMSCs and PBMCs [7, 17, 98]. In comparison, the prevalence of OLP and primary SS (SS not associated to other autoimmune disorders) are reported as 1% and 0.5-1% respectively, within the general population [110, 111]. Autoimmunity in general is described with a female predisposition and the female factor is often mentioned in OLP but recently the gender predispositions are not considered that prominent [111]. However, in SS the female to male ratio is reported as being 9 to 1 [110, 111].

5.1.1 Oral lichenoid lesions

om-cGVHD is clinically diagnosed as a lichenoid-like manifestations with distinctive lesions similar to those for OLP, as defined by the NIH Consensus Diagnosis and Staging Working Group in 2005 and updated in 2014 [5] (Figure 1). Diagnosis of OLP is based on modified World Health Organisation criteria of white bilateral papular or lace-like striations, which could be accompanied by erosive erythema, pseudomembranous lesions and ulcerations [112-114]. The OLP histopathological criteria include a lymphocytic (mainly) band-like infiltrate with liquefaction degeneration. The cGVHD NIH Consensus Pathology Working Group used these lichenoid interface criteria associated with exocytosis and apoptosis to verify an active stage of om-cGVHD [58, 112-114] Following these established clinical and pathological guidelines, both om-cGVHD and OLP should be verified with a final diagnosis showing only consistency, or being diagnostically conclusive for the final diagnosis [58, 113, 115].

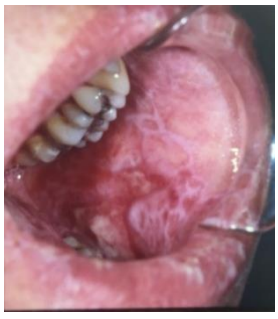

Figure 1. Oral mucosal cGVHD. A clinical image of the left buccal mucosa presenting with extensive lesions considered as severe om-cGVHD. In this picture, diagnostic white reticular lichenoid striations are accompanied with distinctive erythematous and ulcerative features. The buccal mucosa is commonly affected in patients with om-cGVHD. *Photo from 1985, acquired from the archived clinical patient register by Victor Tollemar.*

5.1.2 Oral potential malignant disorders

The spectrum of lichenoid lesions including OLP, and lichenoid reactions associated with drugs and other systemic disorders [115]. Oral potential malignant disorders (OPMD) are a group of conditions that have a verified risk of malignant transformation [114]. The OPMD Working Group revised their consensus report in 2021, showing that OLP, leucoplakia (an unexplained white lesion), erytroplakia (an unexplained red lesion), and om-cGVHD, amongst others should be considered as potentially high-risk lesions with need for personalised management [114, 116]. Long-lasting clinical lichenoid-like reactions commonly display hyperkeratotic plaques, which can be hard to distinguish both clinically and histopathologically from a solid oral leucoplakia [5, 55, 115]. Similar clinical features of lichenoid sclerosus, described in lichenoid skin and vaginal reactions, are uncommon within the oral mucosa due to the lack of scientific literature [115, 117]. Our personal experience from patients attending the Oral Medicine Clinic, University Specialist Clinic, Karolinska Institutet is the diagnostic dilemma and management of late distinctive om-cGVHD manifestations, including hyperkeratotic lesions, which potentially have an increased risk of malignant transformation. A recent case series of om-cGVHD patients with hyperkeratotic plaques observed over time, found these lesions resolved spontaneously, remain unchanged or progressed to secondary oral cancer [118]. An Asian meta-analysis reported secondary solid cancers in HCT-recipients and showed a 16-fold increased risk incidence ratio of oral/pharyngeal cancer post-HCT compared to the general population [20]. It should also be mentioned that a SS diagnosis is associated with an increased risk of developing secondary lymphoma, something which has not been reported in the field of sg-cGVHD [119].

5.1.3 Sjogren's Syndrome-like sicca symptoms

Oral sicca symptoms, as xerostomia and hyposalivation are common in HCT-patients and long-term effects could indicate sg-cGVHD [120-122]. The management and understanding of sicca symptoms post-HCT remain with a key knowledge gap, and therefore the field of sg-cGVHD lacks validated criteria [5, 108]. The experience of elderly individuals and use of polypharmacy display increased sicca symptoms. Furthermore, cancer patients receiving radiotherapy show permanent sicca symptoms, whereas combined irradiation and chemotherapy conditioning; as well as chemotherapy solely, warrants further investigations to fully understand [108]. Current HCT-patients treated at KUH are involved in a multi-centre study addressing these unmet knowledge needs [123].

sg-cGVHD is often considered to affect the MSGs, but major salivary gland dysfunction should be considered possible during both aGVHD, and to some extent in cGVHD [124, 125]. Clinical signs of mucoceles and glandular enlargement, as well as xerostomia are described for both sg-cGVHD and SS, but it is still controversial how these are associated to sg-cGVHD and cGVHD severity [15, 60, 88, 125]. Managing patients with sicca syndrome post-HCT with distinctive signs of mucoceles and xerostomia might lead to the suspicion of a sg-cGVHD diagnosis. Histopathological diagnosis from a labial MSG biopsy could be used to verify the specific histopathological criteria of sg-cGVHD: periductal and acinar infiltrate with ductal damage and acinar degeneration, and fibroplasia in the stroma [58]. In addition, supportive information

involves mapping salivary flow (≤0.2ml/min) with other cGVHD features with particular interest to ocular cGVHD symptoms that includes lacrimal dysfunction (tears ≤1ml/min) [18]. For comparison, diagnostic criteria for SS have evolved over time but were first really accepted in 2002. They were developed by the American and European Consensus Groups in Rheumatology. These standards were further refined by the American College of Rheumatology and the European League Against Rheumatism in 2016 [126]. SS-patients are diagnosed based on ≤0.1ml/min unstimulated whole saliva, a Schirmer`s test showing ≤5mm/5min, ocular staining score of ≥5, labial MSG biopsy with focus score of ≥1/4mm^2 and autoantibodies against SS-related antigen A [126].

5.1.4 Perioral fibrosis

A consequence of persistent inflammation in cGVHD is abnormal wound healing, tissue repair and fibrosis [4]. Perioral fibrosis was for a long time considered to be part of the pathophysiology of skin and oral GVHD, leading to restricted motion of the oral apparatus [55]. The 2014 NIH Diagnosis and Staging Working Group revised the clinical criteria for perioral fibrosis, considering limited mouth-opening to be associated with skin fibrosis, following significant reports where 13% of patients showed both skin sclerosis and limited mouth-opening [5, 18, 58]. Oral fibrosis and sclerosis have been observed within the oral mucosa and salivary gland histopathological profile, but to what extent and functional effect is not fully clear [21, 127].

5.2 PATHOPHYSIOLOGY

Emerging evidence points out that different organ and tissue sites are involved with specific pathobiological processes of cGVHD [98, 128]. Furthermore, early and late cGVHD onset might involve different pathobiological pathways [91]. Description of cGVHD target tissue will most likely direct the pathophysiological models into type of organ structures; exocrine glandular epithelium with dysfunctional lacrimal- and salivary glands, or manifestations of stratified skin and mucosal epithelium [18]. The three-phase biological model (described in section 3.4.2) can be applied to the understanding of oral cGVHD pathophysiology [4]. om-cGVHD inflammatory phase consists of lichenoid erythematous manifestations, which progress with clinically persistent ulcerations and dysregulated immunity [4]. The fibrotic stage might be less prominent for om-cGVHD; however, mucosal manifestations show aberrant healing properties with increased potential for malignant transformation [4, 19, 20]. Furthermore, it should be recognised that sg-cGVHD is mostly related to the late fibrotic process, presenting with degenerated acinar structures, fibroplasia and functional impairment of saliva secretion [4, 129]. Characterisation into biological stages has been demonstrated in patients with limited mouth opening, which display associated features of sclerotic skin cGVHD [4, 18].

A major risk factor for oral cGVHD is the use of PBMCs transplant [128]. Active oral cGVHD show serum components of active inflammation; lower albumin, and higher complement proteins [14, 89, 130]. The mucosal barrier and salivary glands are damaged during conditioning. Oral mucositis is common but oral aGVHD seldomly occurs compared to the gut model, where

mucositis progresses with typical aGVHD [7, 9, 70]. Regarding cGVHD pathogenesis, little is known of the interaction and changes to the microbiota [131]. Low saliva flow rate and xerostomia commonly occur early post-HCT and are prolonged in the event of cGVHD. Saliva from HCT- and GVHD-patients display decreased salivary (s) IgA and increased IgG [129, 132]. Increased composition of albumin, sodium, and anti-microbial proteins such as Lactoferrin were also reported [132-134].

Effector mechanisms in oral cGVHD are similar but not identical to patients with OLP or SS [135-140]. Mononuclear T-cell infiltration is predominant, but differences in location, ratio and magnitude have been observed for all three disorders [135, 137, 141-144]. Also, the role of macrophages, DCs and B-cells differ between cGVHD reports, as well the comparison to OLP and SS. Lichenoid interface histopathology has been demonstrated for om-cGVHD, but terminology and assessment have been inconsistently reported. The T-cell infiltrate target and migrate into the epithelial basal membrane zone, triggering degeneration and keratinocyte apoptosis [142]. In addition, sg-cGVHD histopathology is less explored but has been discussed with similarities to SS histopathology; however, this association remains to be confirmed [129].

Lichenoid skin and mucosal cGVHD is strongly associated to T_h1-, T_c1- and T_h17-cell populations, even though the data supporting oral mucosal and MSG pathogenesis is limited [4, 138, 142, 145]. Type 1 T-cell responses are driven by the IFN cytokines with increased expression of chemokine receptor CXCR3 critical for tissue migration [138, 142, 146]. Infiltrating T-cells (T_h1-, T_c1- and T_{reg}-cells) have been shown to increase in direct proportion to each other [138, 142]. The cytotoxic effect of T_c1-cells is expressed with the granzyme-B and perforin pathway [136, 142]. The cytokine profile of T_h2-cells are typically IL-4 and IL-5, and CCR4, as the chemokine receptor, which have been described in one study associated to both om- and sg-cGVHD infiltrations [138]. Studies into T_h17-cells are few, but evidence suggests a role in the om-cGVHD infiltrate [145].

Immunolocalisation of macrophages in the om- and sg-cGVHD is reported inconsistently, but recent evidence suggests a strong association with om-cGVHD severity [127, 135, 141-144]. DCs have been predominantly described as LCs, but evidence of plasmacytoid-like DC involvement has also been reported in om-cGVHD [127, 135, 137, 142, 147, 148]. Furthermore, increased MHC-II (HLA-DR) expression has been observed on oral keratinocytes, endothelial cells and infiltrating cells in om-cGVHD manifestations [141, 145, 148]. B-cells are rarely found at sites of oral cGVHD compared to patients with SS, but increased circulating autoantibodies are present in patients with cGVHD [131, 142, 144, 149]. There are no verified autoantibodies correlated with type or severity of cGVHD. One study found an association between antinuclear antibodies and oral GVHD, whereas other studies have not [129, 131, 150].

5.3 MANAGEMENT

Patients who develop oral cGVHD experience altered mouth pain and sensitivity to spiced, salty and smoked food stuffs, acidic fruits and vegetables, dressings, carbonated and alcoholic

beverages and oral hygiene products [14, 15, 151, 152]. Some might even have trouble to eat, which in the worst case might lead to nutritional deficiencies requiring hospitalisation [13-15, 89]. Prolonged severe oral cGVHD effects and compromises the quality of life and contributes to early death [8, 13]. Treatment of oral cGVHD aims to alleviate symptoms and heal ulcerative severe lesions [15]. In addition, long-standing cGVHD and immunosuppressive medications have been shown to raise the risk of Candida, bacterial and viral infections, as well as increased risks of developing oral squamous cell carcinoma [13, 15, 20, 153, 154]. The oral specific pathophysiologies remain to be investigated properly as most symptoms and complications have been described relate to oral cGVHD as one clinical entity. However, below we try to distinguish the management based on oral symptoms.

5.3.1 Mucosal manifestations

Lichenoid lesions typically involve white striations, erythema, and/or ulcerations, which lead to pain and increased sensitivity [152]. Sharp teeth should be smoothed, and a soft mouth splint could be manufactured to protect the mucosal surfaces from trauma, particularly if the patients suffer from dry mouth. Treatment involves oral topical ointments or gels of corticosteroids. In Sweden, often the highly potent Clobetasol 0.025% or medium potent Triamcinolone 0.1% are used [15]. Only one randomised clinical trial has investigated the effect of Clobetasol (0.05%) for om-cGVHD [155, 156]. A significant partial to complete response was seen for half of the patients according to the outcomes of the NIH modified OMRS [156]. The use of other immunosuppressants, as topical Tacrolimus 0.1%, showed less effective clinical and histopathological responses compared to topical corticosteroids [127, 156, 157]. However, evidence points out that combination therapy might give some additional therapeutic effects [157, 158]. Topical Tacrolimus ointments need to be monitored for potential altered serum levels, particularly when persisting for longer than two weeks [157]. Severe refractory lesions could also be a target for intra-lesional corticosteroid injection, as well as systemic treatment with Prednisolone [15, 159]. Novel clinical therapeutics involve injection of mesenchymal stromal cells for refractory ulcerative om-cGVHD [160]. Phototherapy including photobiomodulation or photochemotherapy using psoralen and ultraviolet A have also been explored with positive effects [161-163].

5.3.2 Sicca symptoms

Taste dysfunction and masticatory difficulties including swallowing heated, hard, and crunchy foods are associated with oral sicca syndrome [15]. Patients may also experience increased sensitivity and pain due to decreased mucosal integrity [120]. The degenerative glandular infiltration due to severe cGVHD, if prolonged will cause permanent loss of secretory glandular function and therefore early treatment is necessary. Management of dry mouth-related issues include non-prescribed lubricants, to compensate for low saliva function [15]. However, there is conflicting evidence as to whether prescribed topical treatments, such as Clobetasol, improve the feeling of xerostomia or increase saliva flow rates [156, 158]. A soft mouth guard, as described above, could easily benefit the patient’s symptoms. One study tested a dental guard with electrostimulation with potential relief of symptoms; however, large-scale studies are needed

to evaluate the efficacy [164]. Sialagogue therapy, commonly Pilocarpine remains as an option but there is need for close observation for development of any potential pulmonary side-effects [15, 165]. In the case of mucoceles, the effect of topical corticosteroids is non-significant, and surgical removal is seldomly needed due to spontaneous recovery [15, 156].

5.3.3 Perioral fibrosis

The fibrotic pathobiology of cGVHD is poorly described for the oral cavity. Prominent lichenoid reticular lesions may cause mouth stiffness but should be distinguished from perioral fibrosis due to sclerotic skin cGVHD [5, 15]. Lichenoid white striations are otherwise typically non-symptomatic without need for further treatment. Furthermore, manifestations of lichenoid hyperkeratotic plaques have been shown without significant reduction using topical agents of Clobetasol [156]. Altered perioral fibrosis could lead to limited mouth opening, which affects both patients' oral hygiene but also dental treatment resulting in decreased quality of life [15]. Therefore, dental prophylaxis is highly recommended with increased fluoride exposure, close observation, and support from dental professions [15]. Some patients might benefit using a jaw trainer with the aim to widen and stretch the affected tissues [15]. Patients may also feel pain and develop mucosal manifestations related to the fibrotic pathobiology, and therefore need to be closely monitored to distinguish from other tentative causalities [15].

5.3.4 Secondary effects

Maintenance of oral hygiene for patients with ulcerative and sclerotic mucosa, with decreased mouth-opening is difficult. Occurrence of caries, typically at abnormal interproximal and cervical sites, and periodontitis are elevated, and consequently associated dental treatments are technically harder to perform and expensive for the patients who are not always able to work [13, 15, 166]. Long-term survivors with oral cGVHD and concurrent immunosuppressive treatment need careful surveillance for oral cancer development [19, 114, 154].

6 RESEARCH AIMS

The principal aim of this thesis was to explore the diagnostic criteria for oral cGVHD using combined clinical, histopathological, and immune cell characterisation. Specifically, the focus of the studies included in this thesis are:

1. **Histopathological validation (papers I and IV).** Histopathological features of om- and sg-cGVHD, based upon the NIH cGVHD histopathology consultation form and defined criteria were investigated to determine histopathological severity and diagnostics.

2. **Development of chromogenic quantitative immunohistochemistry (paper II).** To develop and validate the use of CellProfiler for quantitative chromogenic IHC staining in comparison to manual subjective assessment.

3. **Assessment of immunopathological profiles (papers III and IV)**. To examine om- and sg-cGVHD immune cell infiltration in a large heterogenous cohort, and determine differences associated to clinical status, severity, pathological diagnosis and duration.

4. **Immunopathological association between om- and sg-cGVHD (paper IV).** To correlate the intra-biopsy immunopathological profile, and to improve our understanding of the association between oral mucosal and MSG involvment in cGVHD.

7 MATERIALS AND METHODS

7.1 RETROSPECTIVE COHORT

The research studies conducted within this thesis used patient material that had already been obtained at the Oral and Maxillofacial Surgery Clinic at KUH and either stored within the archives of Stockholm's Medicine Biobank (SMB) or at the Department of Dental Medicine, KI. The patient material and biopsies were organized into a retrospective HCT-patient KUH cohort (*Figure 1,* **paper I**).

The archived material came from patients referred to the Oral and Maxillofacial Surgery Clinic, KUH due to hematologic conditions with the potential need for HCT. Most of these patient biopsies had been collected post-HCT at clinical follow-up between 1977 – 2011. Some HCT-biopsies and healthy control samples had been collected within the ongoing research project from 2013 and were stored at the Department of Dental Medicine, KI.

The following two sections include the different study protocols with associated ethical permissions, the procedures for biopsy collection at the time, and the workflow leading to the final retrospective cohort in this thesis.

7.1.1 Archived patient material 1977-2011

Study protocol

Patients selected for HCT in 1977 – 2011 were referred to the Oral and Maxillofacial Surgery Clinic at KUH. The clinical protocol involved oral examination prior and post-HCT, and oral biopsies were routinely obtained prior-HCT and at 3-, 6-, and 12-month post-HCT according to the strategy of the Seattle group [22, 141]. Biopsies were further obtained on an individual basis at later time points post-HCT. The dental clinical patient records were archived at KUH along with clinical photographs and related HCT data.

Ethical permission

The Swedish Ethics Review Authority (DNR 2013/1241-31/1) and (DNR 2019-01259) approved the retrospective study of mucosal and salivary gland tissues. The SMB registration ID (BBk 1295) and (BBk 2329) granted permission for the retrieval of archived patient material.

Retrospective review and characterisation

Archived clinical charts were initially reviewed and patients excluded based on defined study criteria. The exclusion criteria are listed below and described with a flowchart in (*Figure 1,* **paper I**):

- Patients recieving autologus stem cells or did not continue with HCT
- Insufficient clinical data or no clinical information >100days
- No biopsies obtained or biopsies only obtained prior-HCT

Study inclusion

Patient biopsies with potential and non-oral cGVHD were included. Potential oral cGVHD patients were included if biopsied when having clinical lesion. The oral biopsies meeting the study criteria were investigated in **papers I, III and IV** based on the identification of a mucosa membrane and/or MSG biopsy.

7.1.2 Patient research material 2013-

Study protocol

HCT-patients from 2013 and onwards have been included within an ongoing research project studying the immune modulatory and regulatory properties in the oral mucosa of patients with oral cGVHD. A standardised 5mm oral buccal mucosal punch biopsy was obtained close to tooth 37/47 in non ulcerative mucosa. Healthy volunteers were included from the Oral and Maxillofacial Surgery Clinic at KUH, or the Orofacial Medicine Public Dental Clinic at KUH during oral mucosal surgery. All patients and healthy controls received and signed informed consent for study inclusion.

Ethical permission

The Swedish Ethics Review Authority (DNR 2012/2235-31/4) and (DNR 2014/1184-31/1) approved the collection of oral mucosal samples to study the immunopathological mechanisms of cGVHD.

Study inclusion

Buccal mucosal samples from HCT-patients were included into **papers I, II and III** for the purpose of histological and immunopathological assessment of om-cGVHD, as well as for the study using CellProfiler software for the quantification of IHC.

Healthy control samples were included into all consecutive papers based on the presence of mucous membrane and/or MSG.

7.2 CLINICAL DEFINITIONS

cGVHD onset and subtype

The retrospective clinical data included onset and diagnostic grade (0-4) of aGVHD. Unfortunately, the data was insufficient to establish if acute symptoms had diminished or persisted at the time of cGVHD diagnosis. Therefore, the reported cGVHD onset was either “de novo” or “prior aGVHD” (the latter symbolising quiescent or progressive onset). Based on the same reasoning, we were not able to classify cGVHD as “classic” or “overlapping” subtype.

cGVHD severity

The overall global severity had been assessed according to established guidelines from the NIH (score 0-3). Information of systemic and topical cGVHD treatment; doses, duration and tapering were inconsistently reported and could therefore not be used as a measurement for

cGVHD activity. Therefore, in **paper III** the research data was associated based on oral mucosal severity (explained below).

Oral clinical status severity

Due to the retrospective nature of the archived cohort and that most patients were prior to the NIH consensus documents, we performed a systematic re-evaluation in **paper I** of oral clinical descriptions and image records for each patient biopsy. Two specialists in Orofacial Medicine with expertise in oral cGVHD from the Oral Medicine Clinic, University Specialist Clinic, Karolinska Institutet in Sweden and the Oral Medicine Unit, Sheba Medical Center in Israel, independently classified the patient samples as "possible cGVHD" or "defined cGVHD". These groups were considered to correspond to distinctive or diagnostic clinical lesions with the aim to verify the final histopathological integrated diagnosis of "possible cGVHD" (evidence of cGVHD but other possible explanations) or "likely cGVHD" (consistent or definitive with cGVHD) according to the NIH criteria. In **paper III,** we further classified biopsies into clinically mild or severe om-cGVHD, based upon the modified OMRS with ulcers and extensive lesions resulting with a severe clinical score [60, 142].

7.3 LABORATORY TECHNIQUES

7.3.1 Histology

We assessed Haematoxylin and Eosin (HE) and Periodic acid Schiff (PAS) histological staining to analyse the mucosal and salivary gland tissues in the study cohort. Salivary gland histopathology was complemented with van Gieson (vG). Biopsies were formalin fixed, paraffin embedded, and sectioned into 4μm thin sections. Of note, in many cases it was possible to withdraw the original pathology stained HE glass slides for the archived SMB biopsies.

Haematoxylin and Eosin (HE)

HE is the routinely applied stain in histopathology. Haematoxylin solutions are partially oxidised, with Haematein as the active ingredient. These solutions are either progressive or regressive based on concentration of dye. Progressive dyes, i.e. Mayer`s Haematoxylin have a lower dye concentration and selectively stain negatively charged nuclear heterochromatin and ribosomal RNA with a strong nucleic acid blue. The counterpart eosin is an acidic dye, which stains charged proteins resulting in pink connective tissue fibres.

Herein, sections were stained with Mayer`s Haematoxylin (Histolab Products AB, Gothenburg, Sweden) for 6 minutes, followed by "bluing" in lukewarm tap water and rinsing in 70% ethanol. Eosin solution (Histolab Products AB) counterstained for 1 minute before dehydration through deionized water, an increasing ethanol gradient into Xylene followed by mounting.

Periodic acid Schiff (PAS)

PAS stains mucopolysaccharides, like proteoglycans and glycoproteins, pink to purplish red.

In the oral mucous membrane, a prominent magenta stain is seen for the reticular fibres, the basal membrane, as well any potential fungal organisms. Whereas mucopolysaccharides in the salivary gland mucous display intense magenta. Haematoxylin adds the strong nucleic acid blue counterstain.

Periodic acid solution (Merck KGaA, Germany) was added for 5 minutes to oxidise glycols to aldehydes, followed with running tap water prior to 15 minutes incubation with Schiff`s reagent (Merck KGaA,). Sections were again rinsed with tap water and then stained with regressive Gill's Haematoxylin III (Merck KGaA) for 2 minutes. Dehydration in alcohols and Xylene was performed before mounting.

van Gieson (vG)

vG stains the nuclei black-brown using an iron-haematoxylin solution, followed by an acid fuchsin that stains collagen red, and cell cytoplasm, including muscle fibres, keratin and erythrocytes with a yellow appearance. The contrast allows suitable interpretation for the assessment of tissue fibrosis.

MSG sections were stained with Weigert's Haematoxylin (Sigma-Aldrich, Germany) for 8 minutes, washed in water and then stained with vG solution (Sigma-Aldrich) for 8 minutes. The slides were then dehydrated in alcohols and Xylene prior to mounting.

7.3.2 Immunohistochemistry (IHC)

Chromogenic IHC is commonly used to visualise the localisation of specific antigens and/or to quantify the magnitude of such immunolocalisation. It is particularly useful to observe in intact tissue to assess disease progression. We employed IHC for immunolocalisation and quantification of the monoclonal surface-trans membranous antibodies listed in Table 2.

Paraffin embedded sections, mounted on Super Frost Plus slides were deparaffinised in Xylene and rehydrated through a series of ethanol's to deionised water and Tris-buffered saline (50mM Tris, 150mM sodium chloride, pH 7.6) with 0.1% Tween®-20 (TBST, Sigma-Aldrich). To expose target antigens, which have been cross-linked with methylene due to formalin fixations, heat antigen retrieval was conducted with basic buffer pH9 (R&D Systems, Abingdon, UK) at ~96^{o}C. To minimize non-specific staining, endogenous peroxidase was removed with 3% (v/v) hydrogen peroxide for 5 minutes and tissue blocking with normal goat serum (DAKO Glostrup, Denmark) and 0.3% Triton-X® -100 (Sigma Aldrich) for 1 hour, before adding the primary antibodies overnight (listed in Table 2). All primary antibodies underwent rigorous titration to determine the optimal concentrations for use.

Table 2. Panel of immunohistochemistry primary antibodies. A list of antibodies used for immune profiling in **papers II, III and IV**, with respect to specificity, isotype, dilution and clone. Manufacturers: DAKO Glostrup, Denmark, and Abcam, Cambridge, UK.

PRIMARY ANTIBODY	ISOTYPE	CLONE (DILUTION)	MANUFACTURER
Anti-CD1a *(DCs/LCs)*	Mouse anti-human IgG1	M3571 (1:15,000)	DAKO
Anti-CD4 *(T_h-cells)*	Rabbit anti-human IgG	Ab133616 (1:8000)	Abcam
Anti-CD5 *(T-cells / B-cells)*	Mouse anti-human IgG1	M3641 (1:300)	DAKO
Anti-CD8 *(T_c-cells)*	Mouse anti-human IgG1	M7103 (1:1000)	DAKO
Anti-CD19 *(B-cells)*	Mouse anti-human IgG1	M7296 (1:150)	DAKO
Anti-CD20 *(Precursors and late B-cells*	Mouse anti-human IgG2	M0755 (1:400)	DAKO
Anti-CD68 *(Macrophages)*	Mouse anti-human IgG1	M0814 (1:30,000)	DAKO

In addition, for all IHC runs, isotype controls (mouse-IgG1 (for CD68) and -IgG2a (for CD20) (DAKO), -IgG1 (for CD1a, CD5, CD8, CD19) (Abcam)) and rabbit-IgG (for CD4) (Vector Laboratories) were included for controls. Biotinylated secondary antibodies (Vector Laboratories, Burlingame, USA, diluted 1:500); anti-rabbit IgG in goat (for CD4) and anti-mouse IgG in goat (for CD1a, CD5, CD8, CD19, C20 and CD68) were added for 1 hour prior to signal enhancement using Vector Labs ABC Elite Kit (Vector Laboratories) for 30 minutes. 3,3´-Diaminobenzidine (Liquid DAB+ Substrate Chromogen System, DAKO) was used as chromogenic substrate for target antigen and development times were optimised for each primary antibody. Sections were counterstained using Mayer's Hematoxylin (Histolab Products AB) for 10 seconds and "blued" with tap water for 6 minutes, prior to dehydration and mounting. All rinses were in TBST.

7.4 IMMUNOPATHOLOGICAL INVESTIGATIONS

7.4.1 Preparation of digital images

Histological and immunohistochemically stained tissue slides were scanned (40x magnification) with a 3D Histech Midi Scanner System (Histolab Products AB) for digital handling. Digital microscopy, for high resolution whole slide imaging (WSI) was performed using PanoramicViewer or CaseViewer software 1.15 (Histolab Products AB). Annotations of the mucous membrane and salivary gland were generated, and the respective images exported from CaseViewer.

7.4.2 Oral mucosal histopathology assessment

Oral mucosal histopathological assessment was conducted on a sample cohort of 303 biopsies obtained from 95 HCT-patients and 15 healthy controls (**paper I**). Inclusion criteria was presence of a complete mucous membrane. To validate the NIH cGVHD histological features, with support from NIH cGVHD histopathology consultation form and published literature, 62 biopsies from 37 of the HCT-patients were randomly selected for screening and calibration [58, 61]. Four histological assessors including one oral pathologist screened the validation cohort for inflammatory infiltrate and exocytosis, lichenoid interface degeneration and apoptosis, including epithelial atrophy and basal membrane alterations. Oral mucosal histological features (NIH cGVHD grading) were classified into six categories, each with severity description ranging from 0 (none/normal) to most severe histopathology (*Table 2*, **paper I**). A weighted pathology score was applied summarising histopathological features into a final pathology score of 0-19. The validation cohort was clustered based on final score into severity grades (G)0-GIV. Subsequently the remaining cohort (199 biopsies) was assessed and graded by the three assessors.

7.4.3 Salivary gland histopathology assessment

Histopathological assessment of MSG tissues was performed on 149 biopsies from 79 HCT-patients and three healthy control biopsies (**paper IV**). Samples were included based on the presences of five lobules or an area of $\geq 1 mm^2$. Validation and calibration were performed by four histological assessors including an oral pathologist, using 25 randomly selected HCT-biopsies and the three healthy controls. The NIH cGVHD histopathology consultation form and defined histopathology criteria was used together with published data to screen for re-occurrent features, and establish severity groups, including peri-ductal and acinar inflammation and exocytosis, degeneration, and fibrosis [58, 61]. Each feature category in the MSG NIH cGVHD grading was scored 0-2, with a total final pathology score of 0-16 (*Table 2*, **paper IV**). Severity clusters were defined to determine final grades (G)0-GIV. The remaining part of the cohort (75 biopsies) was further assessed by the four histopathological assessors.

Furthermore, a second grading scheme was used to validate SS-like histopathology based on the Greenspan composite scheme (score 0-10). This grading module used the Greenspan modified Chisholm and Mason score (0-4) with parenchymal atrophy (0-3) and fibrosis (0-3)

(*Table 3*, **paper IV**) [129, 167]. Calibration was performed on the 28 MSG validation biopsies, followed by whole cohort assessment by the four assessors. Severity clusters and final diagnostic scores (score 0-2) was established.

7.4.4 Computer aided analysis using CellProfiler software

Quantification of IHC exported tissue annotations (30x magnification) was conducted using CellProfiler software (version 3.1.9, www.cellprofiler.org) [168]. CellProfiler algorithms were applied for quantification of chromogenic staining, and workflows were developed for CD4 DAB+ staining as described in **paper II**. In general, the pipeline can be described using CellProfiler algorithms for image preparation, object detection and data output (described in supplementary material for **paper II**):

1. Image preparation (Color to grey, Image math, Rescale intensity):
   The raw images were converted to greyscale with increased intensity between occupied and unoccupied regions. The rescale intensity allowed a scale of 0-1 for the application of thresholds for further identification.

2. Detection of objects (Unmix colors, Identify primary objects, Mask image):
   Primary objects in the forground were detected including white spaces within or surrounding the biopsy, haematoxylin and DAB+ stained regions. The white regions were added as masked areas in the downstream analysis of stained regions. Unmix colors identified objects based on the dyes haematoxylin and DAB+.

3. Data output (Filter objects, Objects overlay):
   CellProfiler software has the capacity for numerous outputs including numbers of DAB and/or Haematoxylin stained cells, Haematoxylin-stained nuclei overlayed with DAB+ staining (DAB+ nuclei), DAB+ stained regions, DAB+ stained pixel area, and DAB+ stained pixel area per total area (Figure 2). Data measurments used for biopsies in **paper II** included DAB+ stained nuclei, whereas the CellProfiler output for **papers III and IV** included DAB+ stained pixel area on white masked tissues area.

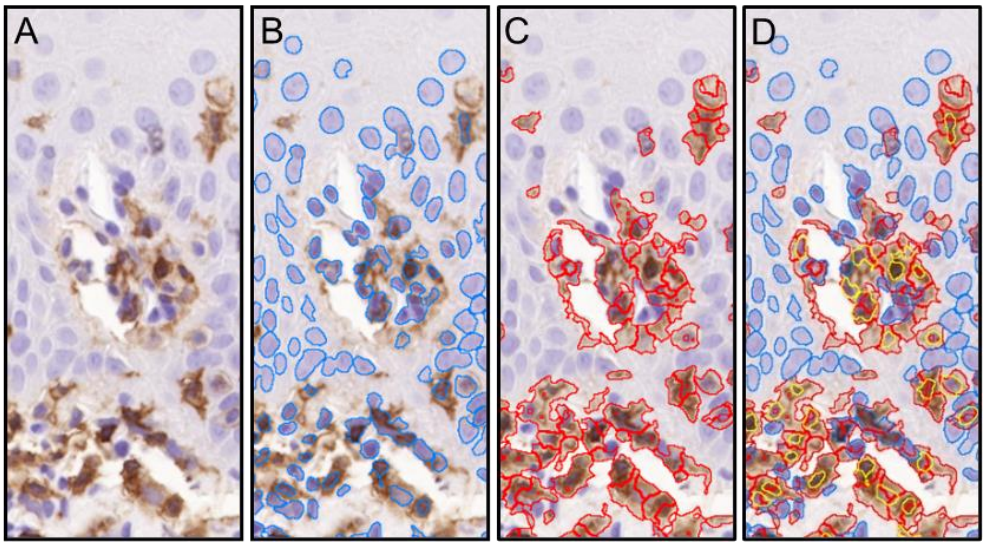

Figure 2. Quantification of chromogenic immunohistochemistry using CellProfiler software. Images with CD4 DAB+ stained oral mucosa membrane segments. A) Raw CD4 DAB+ stained image, B) Detection of haematoxylin-stained nuclei (blue), C) detection of DAB+ stained regions (red), and D) Image with quantified object overlay representing DAB+ nuclei (yellow). *Cropped images acquired from CellProfiler data output, illustration by Victor Tollemar.*

7.4.5 Comparison of quantitative IHC methodology

Ten om-cGVHD patient biopsies and five healthy control biopsies were included to make a comparison between manual counting and CellProfiler software image analysis (**paper II**). A total of 299 images with CD4 DAB+ staining were annotated at 30x magnification and exported from PanoramicViewer. A protocol was developed to clarify limits for positive/negative manual detection. Haematoxylin-stained nuclei overlayed with DAB+ staining (DAB+ nuclei) not touching the image border were included as positive cells. Four assessors including one oral pathologist manually counted the immune-positive DAB+ staining using the numerical counting tool in Adobe Photoshop® (Adobe Systems Incorporated, San Jose, CA). The four assessors were calibrated on an image set of 45 randomly selected images and concordance was established. The remaining cohort (254 images) was divided and subsequently counted manually by one of the three assessors. Data from the whole cohort of 299 images included manually counted cells, and multiple CellProfiler outputs. Measurements between manual counters and CellProfiler were correlated, and inter-platform agreement determined for CellProfiler software.

7.4.6 Oral mucosal and salivary gland immune cell profiling

Immunopathological profiling of the oral mucosa and MSG was conducted in **papers III and IV**. Biopsies meeting the histological inclusion criteria post further sectioning were investigated. 170 oral mucosal and 68 MSG biopsies were immunohistochemically stained with the panel of antibodies listed in Table 2, and the resulting slides were digitised. The DAB+ stained WSI, was annotated and segmented using ImageMagic software (version 7.0.8, www.imagemagick.org), to 1000x1000 pixels (>15kb) for subsequent analysis in CellProfiler.

7.5 STATISTICS

Data analysed within **papers I, III and IV** were mainly quantitative and analysed using non-parametric statistics. In **paper II**, the sample cohort for the quantitative IHC data, with images of positive staining, were normally distributed. Statistical p-values were determined for a given test to determine whether the data were consistent, or inconsistent with the null hypothesis. p-values ≤ 0.05 were considered as significant. To estimate the certainty that our sample data reflected true population data, 95% confidence intervals were given. All analyses were conducted in Prism 8 (GraphPad Software, La Jolla, CA) if not stated differently.

7.5.1 Paper I

Pathology scores were visualised in scatterplots to define classes along the scoring range. To determine pathological classification cut-offs, Jenks natural breaks for one-dimensional data was applied to define boarder values between the groups [169].

Agreement for categorical clinical evaluation and histopathological assessments were tested using Cohens weighted kappa [170]. A value of ≥ 0.6 was considered a substantial agreement. Non-parametric analysis of variance, Kruskal Wallis test, with Dunn`s correction was used for multiple comparisons between histological scores. Data was presented with box-whisker plots for median, interquartile-, minimum- and maximum range, within the individual patient groups.

To test the probability of predictive histological feature scores on clinical groups, we used receiver-operating characteristic curves. Area under the curve was calculated as an estimate to explain how often a random selection of diseased individuals presented with a higher test value than non-diseased patients. Values between 0.5 to ≤ 0.7 were considered less accurate than 0.7 to ≤ 0.9 that were moderately accurate. The likelihood ratio (LR) was assessed to express how histological features changed in odds for diagnosed patients with a positive test. LR combines the proportion of biopsies with true positive (sensitivity) against true negative (specificity), as a simple measurement of the probability of a disease given a positive test. Post-test estimates for probability were considered; LR >10 as a high likelihood that disease was present, LR of >5-10 moderately and LR of >2-5 low [171].

7.5.2 Paper II

The statistical analysis within this paper was performed in Stata version 16 (StataCorp, College Station, TX). Manual counter concordance, and comparison between manual counts and CellProfiler, were displayed using correlation scatterplots with linear association and fitted simple linear regression. The intraclass correlation was applied to measure reliability of the intra-image counting using one-way analysis of variance. The agreement between manual counting and CellProfiler output was plotted with assessor differences against the mean, according to the Bland-Altman model [172]. Strong agreement was considered if $\geq 94\%$ of data points laid within the $\pm$ 1.96 standard deviation.

7.5.3 Paper III

Quantitative IHC was analysed based on marginally predicted mean pixel area, determined using generalised estimated equations with an independent correlation matrix to account for potential intrasubject correlation [173]. The statistical model was calculated with Stata version 16 (StataCorp, College Station, TX). Comparison of the mean pixel area was presented for each individual patient group as fold-change compared to healthy or NIH cGVHD G0, which was normalisied to 1. Pairwise comparisons included the mean pixel area ratio between individual patient groups. For categorical data of clinical and pathological severity, Fischer's exact test was used to determine distribution [174].

7.5.4 Paper IV

Association and concordance were determined for histological assessors, histopathological features, grading module outputs and the intra-biopsy association between the oral mucosa and salivary gland, using Spearman's mean rank correlation. The coefficient of correlation was considered strong with a value ≥0.7. Pathological classification was determined with scatterplots supported by Jenks natural breaks as described above for **paper I**. We tested the agreement using Cohens weighted kappa for histological assessors and pathological diagnostics between grading module outputs and the intra-biopsy association. Quantitative IHC was analysed and marginally predicted mean differences compared for the pathological diagnostics as describe for **paper III**, using NIH cGVHD G0-G1, and Greenspan composite score 0, normalised to 1.

7.6 ETHICAL CONSIDERATIONS

Patients going through HCT and that subsequently might develop cGVHD, are individuals with a high disease burden. Their medical struggle fighting their disease with prolonged treatment and possible complications are tremendous. The medical visits are often multiple, and many are involved with other research trials, and as a consequence it is not always possible to participate in various research projects. It is admirable that these patients volunteer to medical science, for the benefit of future patients.

Research conducted within this thesis followed the Declaration of Helsinki and approval was obtained from the Swedish Ethics Review Authority (described in sections 7.1.1 and 7.1.2). Research participants from 2013 and onwards were included with biopsies taken by a clinical research investigator during routine care. These patients were given written and oral study information including aim and purpose of their inclusion, research procedures and potentially risks with participating, and the legislation associated with sensitive information (EU General Data Protection Regulation-GDPR; 2016:679), storage of samples into a biobank (Biobank Act 2002: 297), and the potential use of research material in future projects (Ethical Review Act 2003: 460). Patients that volunteered to participate signed informed consent.

The majority of patient samples within the research project had been obtained prior to 2013 and were registered and stored at SMB (914). Associated clinical information and HCT-data had been registered at KUH. Through ethical approval, register patient information and samples were possible to use in research without written informed patient consent. However, patients always have the right to withdraw their information and samples from registers, at any time point.

There were some ethical considerations associated for study inclusion in clinical routine practise. The physician taking care of patient's health care was involved in the research project, and as such might hypothetically affect the patient's decision to participate. It is also an ethical dilemma that patient might feel obligated to participate with worries that it otherwise might affect their future routine health care visits. A strength of the 2013-onwards cohort was that the informed consent, included the patient's right at any point to drop out without any reasons. It is also clarified that participation was voluntary and that their decision did not affect health care received.

Benefits of retrospective research projects include the use of patient information and materials already collected. Even though oral mucosal biopsies are considered minimally invasive, and have properties of scarless wound healing, there are always risks of complications including bleeding, infections and pain. Retrospective samples and register data need careful handling for integrity of data and traceability of personal data.

8 RESULTS AND DISCUSSION

Research results presented and discussed within the following sections explore the diagnostic criteria for oral cGVHD using combined clinical, histopathological and immune cell characterisation. The data refers to the thesis **paper(s) I:** Tollemar., et al. Biol Blood Marrow Transplant 2020, **paper II:** Tollemar., et al. Cytometry A 2018, **paper III**: Tollemar., et al. Manuscript 2022, and **paper IV:** Tollemar., et al. Manuscript 2022.

8.1 COHORT

Large cohort analysis has been missing in the field of oral cGVHD, and as a consequence the field has struggled with vague study criteria and definitions. The observational cohort investigated in this thesis has been one of the largest to date. In **paper I**, the retrospective patient cohort (1977-2011) included 789 patient records and were initially screened to define patients treated with HCT with clinical records covering >100 days post-HCT. 300 patients fulfilled the inclusion criteria. Patients without biopsies taken post-HCT were excluded, as well as potential oral cGVHD patients that only had biopsies prior/post clinical manifestations. The final SMB cohort consisted of 65 potential oral cGVHD patients with 195 mucosal biopsies, and 30 randomly selected non-oral cGVHD patients with 76 biopsies. Additionally, 17 HCT-patients and 15 healthy control biopsies were included from the research cohort (2013-onwards). The retrospective KUH cohort of this thesis included 95 HCT-patients and 15 healthy controls, with a total number of 303 biopsies.

Overall, the HCT-patient cohorts (**papers I, III and IV**) were considered treated with conventional HCT regimes, and most patients were transplanted between 1977-1991 (**papers I, III and IV** respectively: n=73;65%, n=63;67% and n=66;82%). The conventional HCT regimes involved MAC (n=88;79%, n=72;77% and n=70;86%), BMSC infusion (n=89;79%, n=74;79% and n=76; 94%), and single-agent or combination of Cyclosporine and Methotrexate as GVHD prophylaxis (n=98;88%, n=82;87% and n=74;92%). Oral cGVHD is common following both BMSC and PBMC infusion, but PBMCs seems to have an even higher risk for oral cGVHD development [17, 128]. RIC is today commonly used and relies more on GVL, compared to our study cohorts treated with MAC. Both oral mucosa and the MSGs are damaged due to MAC irradiation, with potential long-term effects on glandular tissue [108, 175]. Two patients in the study cohort developed oral mucosal cancer after prolonged om-cGVHD manifestations, a well-reported risk following morbid forms of om-cGVHD [85, 176].

The study cohort included into **paper II** constituted 10 randomly selected om-cGVHD patients from the research cohort (2013-onwards), who were considered treated with modern HCT regimes, including PBSCs and higher numbers received RIC. Five randomly selected healthy controls were also included into the study.

8.2 CLINICAL ASSESSMENT

Oral cGVHD clinical criteria has evolved over time, and the NIH Consensus documents were developed to standardise the characterisation in clinical trials [6]. However, few cohort studies have been published with the updated oral cGVHD clinical definitions [14, 18, 127, 142, 146]. In **paper I**, a systematic re-evaluation was performed for clinical definitions according to the established clinical NIH cGVHD criteria [5]. From the retrospective patient cohort (1977-2011), 230 SMB biopsies fulfilled the histological inclusion criteria. 31 biopsies were allocated to clinical consensus discussion due to limited patient information. Two Orofacial Medicine Specialists individually assessed the remaining 199 SMB biopsies to allocate the clinical manifestations as definitive om-cGVHD (diagnostic lichenoid om-cGVHD), possible om-cGVHD (distinctive om-cGVHD) and oral HCT controls (healthy mucosa). The two clinicians were found with a strong, almost perfect agreement, as displayed with weighted kappa statistics of 0.81. It is recognised that a degree of oral cGVHD clinical inter-rater variability is common and depends on clinical experience and type of clinical lesions [87, 88, 177]. Biopsies without clinical agreement, and those with limited clinical information were discussed in consensus, resulting in the exclusion of 49 patient samples due to insufficient information to meet the clinical study criteria. Due to the retrospective design, we were not able to classify the NIH oral diagnostic criteria (score 0-3) based on limited information on food intake and symptoms [98].

cGVHD clinical therapeutic evaluation assessed by NIH OMRS (score 0-12) has recently been used to characterise cGVHD cohorts [88, 156, 157]. Our research cohort (2013-onwards) had been clinically scored with a modified version of the original OMRS [86]. Biopsies with an OMRS score were therefore designated to the definitive om-cGVHD study group and not involved in any clinical re-evaluation.

8.3 HISTOPATHOLOGICAL GRADING

The terminology, and transparency of histopathological evaluation has shown great variance across immunopathological studies [127, 138, 178], and therapeutic intervention trials [157, 163]. Most commonly, samples are included based on study specific interpretations of the NIH histological criteria for oral mucosa (lichenoid interface lymphocytes with exocytosis and variable apoptosis), and MSGs (periductal lymphocytic infiltrate and damaged intralobular ducts, fibroplasia in periductal stroma, mixed lymphocytic and plasmocytic inflammation with acinar destruction) [58]. Hypothetically, this approach comes with the risk of only studying the most severe patients, or that the interpretation of the NIH histological criteria allows a wide range of heterogenous biopsies [157].

8.3.1 Oral mucosal histopathology

Of the initial 303 biopsies in **paper I**, 212 were included following exclusion based on histological and clinical criteria not being traceable in SMB (n=23), insufficient or poor tissue histology (n=19), or not fulfilled clinical criteria (n=49). The clinical classification of biopsies

can be found in Table 3 (section 8.5). Published om-cGVHD literature was reviewed and using the calibration cohort (n=62 biopsies) screening for re-occurring histological features was performed. Learning from the previous grading methods according to Sale et al, and Horn et al, compiled categories with multiple features were avoided if possible [22, 109, 178, 179]. A NIH cGVHD histopathology consultation form had been published by the NIH Consensus Pathology Working Group, and along with the minimal criteria for OLP and om-cGVHD we formalised an oral mucosal grading module based upon six feature categories (Figure 3) (*Table 2,* **paper I**) [58, 61, 113].

Inflammation infiltrate (score 0-4)

Lichenoid infiltrate has been classically described as a lymphocytic band-like aggregate associated close to the basal membrane region [180]. It is not fully clear whether there are differences in the pathogenesis between OLP and om-cGVHD, or if systemic immunosuppressive therapies given to om-cGVHD patients might interfere with the pattern of lichenoid interface inflammation [115, 137, 138]. However, in our cohort only 15% presented with classical band-like structure. A range of inflammation was observed, from sparse (33%) to tight clusters (19%), that progressed into band-like inflammation, which was extensive for the most severe cases (Figure 3A). In diagnostic om-cGVHD, inflammation was found with a high LR (13-fold, $p<0.0001$) compared to prior HCT, whereas the LR for band-like infiltrate was considered relatively low (4-fold, $p\leq0.0005$) in comparison to oral HCT controls.

Exocytosis (score 0-3)

Intra-epithelial lymphocytes have not gained much focus in the classification of OLP but are recognised as a key feature in om-cGVHD histological activity [58, 143]. Levels of exocytosis were found in variation: sporadic (38%), focal (19%), and widespread (10%) (Figure 3B). The NIH cGVHD histopathology consultation form specified guidelines of ≥5 cells/10x field of view, which could be applied to determine the limits of focal or widespread pattern [61]. The LR for detected widespread exocytosis in diagnostic om-cGHVD was considered low (3-fold, $p\leq0.0001$) compared to HCT controls. Of note, intra-epithelial lymphocytes were detected even though the magnitude of inflammation was reduced, which might suggest persistent effector activity or that tissue resident T-cells might be involved in the pathogenesis [58, 181].

Liquefaction degeneration (score 0-3)

Epithelial cGVHD damage was initially described as necrosis, but features of hydropic degeneration, vacuolisation, spongiosis, and squamatisation have been defined [22, 109, 139, 144, 148, 157, 178]. On a cell-level, signs of atypia and disarray have been reported [157, 178]. We appointed the processes occurring as liquefaction degeneration, a distinct feature in the criteria of OLP [112, 113]. Indeed, all terminologies above could occasionally be identified, but using liquefaction degeneration as a composite category, we found sporadic (30%) initial signs of basal cell vacuolisation and spongiosis. Widespread liquefaction degeneration appeared along the basal cell layer and was observed for 15% of biopsies. For the most severe cases, the epithelial connective tissue interface showed complete degeneration with confluent areas of liquefaction

and squamatisation (19%) (Figure 3B). However, the LR for liquefaction degeneration in diagnostic om-cGVHD was low.

Use of different terminologies might reflect study specific characterisation highlighting the pattern of severity, with associated risk for malignant transformation [19]. However, to evaluate such risk, stringent use of dysplasia grade remains the only predictive risk factor [113, 114, 116]. Liquefaction degeneration has further been described both in OLP and ocular cGVHD, as a process related to epithelial mesenchymal transition (EMT) [182, 183]. During EMT, epithelial cells lose their cell-cell adhesion polarity and express a phenotype associated with mesenchymal cell motility, which is shown to be associated with carcinogenesis, wound healing, fibrosis and could potentially be prognostic for dysplastic lesions [184, 185]. This needs further investigations since no studies for om-cGVHD have assessed markers for EMT, such as cadherins, catenins, vimentin and laminin-5 [184].

Apoptosis (score 0-2)

Programmed cell death is the hallmark for cGVHD activity [58, 186]. In the field, apoptosis has been mentioned in terms of apoptotic-, eosinophilic-, Civatte-bodies, and dyskeratotic cells (Figure 3E and F) [22, 109, 139, 142, 144, 148]. However, the amount of apoptosis has been reported inconsistently [127, 142, 144, 178]. The NIH cGVHD histopathology consultation form suggested a cut-off of ≥1 apoptotic cell/10x field of view to decide the extent of cell death, however this has been questioned as a reliable measure [61, 178]. The amount of apoptosis has been reported in OLP to not necessarily be of quantitative importance [187]. We found patient dependent variations, with some presenting with sporadic (30%) and widespread (15%) features. Minimal sporadic apoptosis was seen with a moderate (9-fold, $p<0.0001$) LR between diagnostic om-cGVHD and prior HCT, but low LR for widespread apoptosis (2-fold, $p<0.005$) compared to HCT controls. In OLP, it has been suggested that apoptosis and liquefaction degeneration are two separate processes of keratinocyte destruction [187].

Basal membrane alterations (score 0-1, and 4)

Basal membrane alterations including thickening, partial clefts, and Max Joseph separation, have been observed in both OLP and om-cGVHD histopathology [109, 144, 178, 188]. Increased thickness of the basal membrane was found in 30% of patient samples. This feature has been associated with clinically erosive OLP [189]. Max Joseph space would typically indicate ulcerative manifestations, whereas branching and disruption have been observed with various lichenoid and leucoplakia lesions, and might reflect aberrant wound healing and EMT [185, 189, 190]. Epithelial detachment, and/or the formation of pseudo rete ridges were defined as the most severe features (Figures 3C and D). This was found in 12% of the sample population and was considered with a moderate LR (5-fold, $p<0.0001$) in diagnostic om-cGVHD compared to HCT controls.

Epithelial flattening and atrophy (score 0-3)

Flattening of rete ridges was determined across the biopsy area of study specimen: <25% (found in 34%), 25-75% (found in 26%), and a flat atrophic epithelium was observed in 21% (Figure 3G). Diagnostic om-cGVHD was found with a moderate LR to present with a complete

atrophic epithelium (8-fold, p<0.0001) compared to prior HCT, but considered of low LR compared with oral HCT controls. The oral epithelium might indeed be hyperkeratotic or acanthotic but based on the outcomes from our study it was not considered as important to define active criteria for om-cGVHD progression [61].

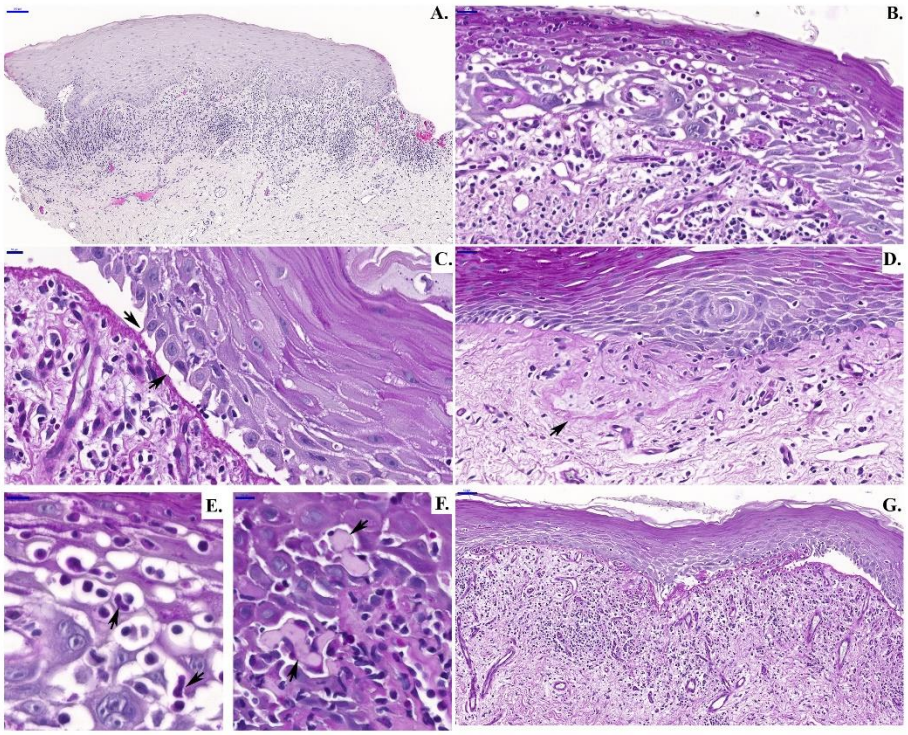

Figure 3. Histological images of oral mucosal histopathological features. A) Haematoxylin and Eosin-stained oral mucosa present with extensive band-like inflammation (scale bar 100µm). Periodic acid Schiff oral mucosal histology represent, B) Widespread exocytosis and confluent liquefaction degeneration (scale bar 20µm), C) Basal membrane separation where epithelial cells loose attachment (arrow) (scale bar 10µm), D) Alterations of basal membrane pseudo rete ridges (arrow) (scale bar 10µm), E) Apoptotic eosinophilic body (scale bar 10µm), F) Civatte bodies (scale bar 10µm), and G) Atrophic oral epithelium with flattened rete ridges across the biopsy (scale bar 100µm). *Image published with permission from Elsevier, Tollemar., et al. Biol Blood Marrow Transplant 2020.*

8.3.2 Oral mucosal histopathological diagnostics

The complete cohort was histopathologically graded according to the above-described feature scores, with a total scoring range of 0-19. Severity classes were determined, allocating each biopsy into grades (G)0: 0-2, GI: 3-5, GII: 6-9, GIII: 10-13, and GIV: 15-19. The histopathological assessors who individually graded the cohort showed substantial agreement (k: 0.71, 0.78 and 0.84 respectively). Overall, the most severe feature score for every category was associated with the final grades of GII-GIV and patients post-HCT, although atrophy was found to be an exception. Findings of sub-clinical cGVHD, as found in oral HCT controls, has been reported previously [191-193]. The association to clinical groups was found accordingly (*Figure 3*, **paper I**): healthy (median score 1, G0), prior HCT (median score 2, G0), oral HCT

controls (median score 4, GI), distinctive om-cGVHD (median score 7, GII) and diagnostic om-cGVHD (median score 10, GIII). All samples post-HCT showed significantly higher scores compared to healthy and prior HCT, but only diagnostic om-cGVHD scores were significantly larger compared to the scoring of oral HCT controls ($p<0.001$).

We compared the pathology scores for cofounding factors. All clinical groups were tested for the influence of CMV reactivation, but no differences were noted in the pathology scores of our patients. Other factors, such as the overall global cGVHD diagnosis (score 0 or 1-3), and for oral HCT controls the influence of future om-cGVHD was investigated. We did not find any statistical differences within our study population of om-cGVHD and overall grade (score 0 or 1-3). The oral HCT controls did not predict future oral cGVHD specifically, but the oral HCT controls with overall cGVHD (score1-3) presented with significantly increased pathology scores compared to those without cGVHD. In addition, the whole sample cohort was characterised based on global cGVHD diagnosis (score 0 or 1-3) and time post-HCT (*Figure 3*, **paper I**). Biopsies with a global cGVHD diagnosis (score 1-3), obtained within the first year of HCT, showed significantly higher scores than biopsies with no cGVHD (score 0). Biopsies prior cGVHD and past the first year also presented with increased pathology but it was not found with statistical significance. This finding highlights that global activity does indeed influence the activity in the oral mucosa, but this might only be significant at onset and early disease stages [191, 192].

We applied the NIH minimal histopathology criteria as: score ≥2 for inflammation, score ≥1 for exocytosis, and score ≥1 for apoptosis [58]. Based on our findings, liquefaction degeneration was a common feature and showed similar LRs as exocytosis and apoptosis. Furthermore, we identified liquefaction degeneration as a suitable feature for the minimal oral cGVHD criteria, in similarity to the criteria for skin cGVHD and OLP pathology [58, 113]. Severe basal membrane alterations were considered with highest LR and warrants further investigation as a potential biomarker. Using the defined limits for the NIH histopathological criteria we found 18% of oral HCT controls (GII-GIV), 34% of distinctive om-cGVHD (GII-GIV), and 45% of diagnostic om-cGVHD (GII-GIV) to present with active pathology. Hence, suggesting the NIH cGVHD final diagnosis could be determined accordingly: G0-GI ("no/inactive cGVHD"), GII ("possible cGVHD") and GIII-GIV ("likely cGVHD").

8.3.3 Minor salivary gland histopathology

In **paper IV**, 250 SMB patient samples were retrieved and investigated for inclusion into MSG histopathology assessment. 146 biopsies included MSG segments and were assessed for the study purpose. 46 samples were excluded based on histological inclusion criteria (n=44) or due to other pathological processes (n=2). A validation cohort of 25 MSG biopsies and three healthy controls were assessed to characterise the NIH cGVHD grading (*Table 2*, **paper IV**). In addition, the same cohort was employed for the assessment of the Greenspan composite score (*Table 3*, **paper IV**) [58, 61, 129]. The NIH cGVHD grading assessed ductal- and acinar regions separately based on mild/focal, or marked/widespread; inflammation, exocytosis, destruction and fibrosis (Figure 4):

Inflammation

Periductal and acinar lymphocytic inflammation is considered specific for cGVHD [58]. However, a plasmocytic infiltrate could also be present, as well as the formation of SS-like focused lymphocytic clusters [58, 129]. Overall, inflammatory components were found with a mixed infiltrate involving both plasma cells and lymphocytes [144, 178]. We observed an overall similar distribution with peri-ductal (mild: 52%, marked: 32%) and acinar inflammation (mild: 55%, marked: 27%), as reported by others (Figure 4A and B) [178]. The inflammatory Greenspan modified Chisholm and Mason score (1-4) was found with a similar distribution, with almost half of the biopsies displaying ≥1 foci/4mm[167]. The close association between peri-ductal and acinar inflammation resulted in a diffuse widespread pattern rather than typical foci [139, 178]. Despite this observation, a large proportion of samples were recorded with a focus-score, but this was probably dependent on the disease specificity, severity, and duration post-HCT [122, 129]. It is interesting to note, that assessment of foci in SS-patients showed considerable inter-rater variability and high potential risks for false results when using histological assessment over IHC [194]. The question remains whether there is a need to separate the grades for ductal and acinar infiltrate, if the focus-score should be considered specific and what effect does conditioning and treatment have on the degree of false positive inflammation [58].

Exocytosis

The NIH cGVHD grading specifically included exocytosis as a feature with periductal and acinar inflammation, in contrast to the Greenspan composite score. [58, 129, 143, 178]. We deviated from the NIH feature criteria, as the combined (inflammation and exocytosis) feature category lacked the impact of assessing exocytosis separately, to understand cGVHD activity [179]. The assessment of exocytosis in the ducts and acini was found as mild/focal (30% and 27% respectively), and as marked/widespread (15% and 2% respectively) (Figure 4D and E). The identification of lymphocytic acinar migration with exocytosis was hard to assess histologically. Widespread exocytosis was particularly low in the acini, which could be attributed to heavy inflammation leading to acinar destruction and subsequent difficulties in assessing acinar exocytosis.

Destruction

Signs of glandular destruction with reduced PAS+ area were commonly detected but previous studies have shown variable distribution [122, 144, 178]. In contrast to the exocytosis feature category, destructive features like vacuolisation, atrophy and apoptosis were combined as a single score. Apoptosis is described with variation in SS, but potentially associated with late stages of the disease [149]. We assessed the NIH cGVHD grading based on focal- (30%) or widespread ductal damage (15%), commonly based upon vacuolisation (Figure 4D). Acinar degeneration was found with focal (44%) or widespread features commonly ductal metaplasia (29%) (Figure 4A). At the same time, the Greenspan composite score evaluated area of parenchymal atrophy (score 1-3), which could be more comparable to the NIH cGVHD acinar degeneration. The Greenspan score presented with score 1 (44%), score 2 (30%) and score 3 (14%). Destruction is a key-feature, along with inflammation, but it can also result from conditioning and drug burden [58, 122].

Fibrosis

Interstitial fibrosis, typically in combination with acinar destruction, has been reported to play an important role in the histopathological grade [58, 178]. Assessment and interpretation of fibrosis need to consider the extent and/or ECM density, as few signs of fibrosis might indicate a false positive finding [144, 178]. In particular, fibrosis in the MSG is linked to elderly people, and potentially non-specific features following conditioning [58, 122, 129]. Indeed, the NIH cGVHD grading was found in our cohort to present with high levels of mild fibrosis, both in the ducts (66%) and interstitially (58%). However, using the NIH cGVHD grading, marked peri-ductal fibrosis was only found in 6% of biopsies, whereas 20% of patient samples displayed marked interstitial fibrosis (Figure 4A and C). The Greenspan composite fibrosis score (score 1-3) was also considered relatively low (score 1; 44%). It should be questioned whether these findings could be non-specific, due to previous conditioning rather than cGVHD pathogenic.

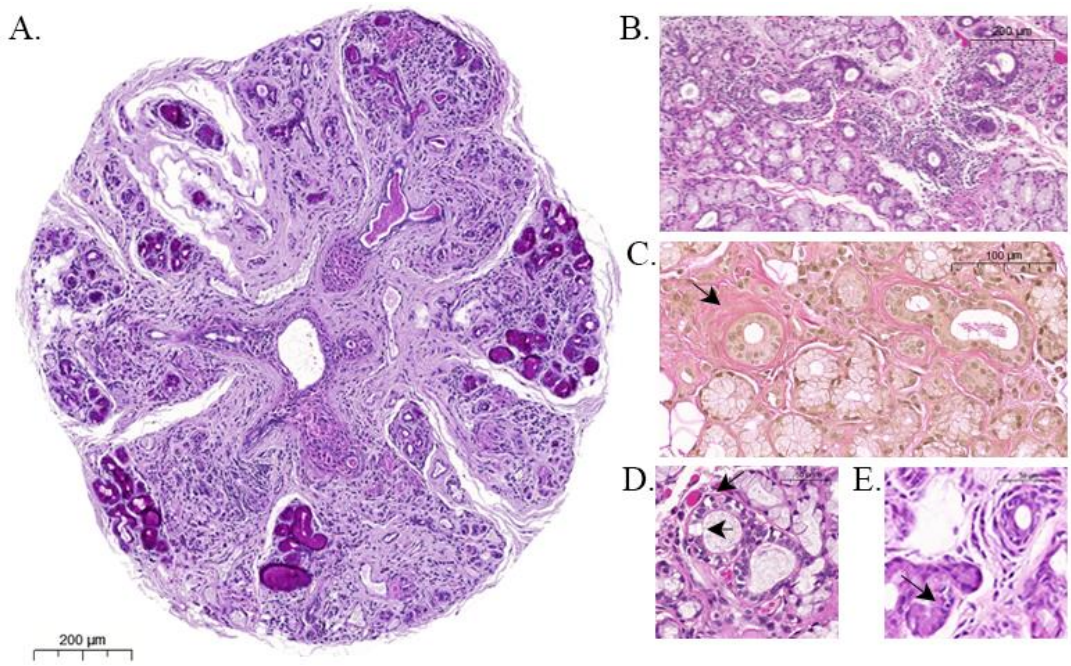

Figure 4. Histological images of minor salivary gland histopathological features. A) WSI of the MSG histopathology stained with Periodic acid Schiff, demonstrates inflammatory infiltrate within the degenerated acinar units and interstitial fibrosis (scale bar 200µm), B) Haematoxylin and Eosin-stained ductal area with signs of peri-ductal infiltrate (scale bar 200µm), C) Observed peri-ductal fibrosis using van Gieson staining (black arrow) (scale bar 100µm), D) Intra-ductal lymphocyte exocytosis and vacuolisation (arrow) (scale bar 50µm), E) Exocytosis into acinar (arrow) and ductal cell units (scale bar 50µm). *Figure from* ***paper IV****; Tollemar., et al. Manuscript 2022.*

8.3.4 Minor salivary gland histopathological diagnostics

Whole cohort grading using the NIH cGVHD grade and Greenspan composite score was found to have a strong correlation between the histological assessor and the oral pathologist (NIH cGVHD grading r 0.79, and Greenspan composite score r 0.83). The intra-grading comparison between the two grading methods also revealed a strong correlation (r 0.90). We determined diagnostic classes for final pathology scores for the two grading methods respectively. Cut-offs were established and biopsies were allocated into NIH cGVHD grades (G)0: 0-2, GI: 3-4, GII: 5-7, GIII: 8-11, and GIV: 12-16, and Greenspan composite scores: score 0: 0-2, score 1: 3-6, and score 2: 7-10. The NIH specific MSG histopathology criteria have previously been assessed but without clear criteria for a final diagnosis [143, 178]. Features of peri-ductal and acinar inflammation and exocytosis, destruction of ductal and acinar cells, and subsequent fibrosis remain important to evaluate. However, close considerations are needed to verify the specificity of these features [122, 129, 143, 144, 178]. The criteria applied for the NIH cGVHD grading was considered ≥2 for ductal and/or acinar inflammation, as well as ≥2 for ductal damage and/or acinar degeneration [58]. Greenspan composite criteria was validated as Greenspan score of ≥2 with an additional score of ≥1 for combined atrophy and fibrosis [129]. Both modules allocated the most severe grades, n=38; 100% of GIII-GIV ("likely cGVHD") and n=33; 100% of Score 2 ("likely GVHD") respectively, with n=4; 17% of GII ("possible cGVHD") and n=21; 38% of Score 1 ("possible cGVHD"). As such, we are comfortable to say that both modules identify patients with evident signs of pathology. The strict feature criteria applied by NIH cGVHD grading was found to be more specific, resulting in less samples diagnosed as "possible/likely cGVHD".

8.4 QUANTIFICATION OF IMMUNOHISTOCHEMISTRY STAINING

Digital pathology and computer supported analysis have increased rapidly over the years [195]. This expansion relates to improved, standardised, and repeatable quantification in respect to the manual assessment [196]. CellProfiler software have been used in multiple reports for the quantification of fluorescent staining, but the automated application for chromogenic IHC remains to be validated [168, 197-199]. Assessors for manual counting were calibrated on the same image set of CD4 DAB+ staining, and the concordance showed a strong coefficient of determination (r^2 >0.91) between the manual assessors (*Figure 2*, **paper II**). The concordance was surprisingly good, as inter-rater variability remains as major issue in pathology [195]. Unexperienced counters often need training to establish concordance, as well as robust grading criteria to lower the risk of errors [195]. Comparison between manually counted cells and the different outputs from CellProfiler were assessed (*Figure 3*, **paper II**). Coefficient of determination showed strong values for detected DAB+ cells and DAB regions of r^2 0.938 and r^2 0.927 respectively. For these CellProfiler outputs, ≥94% of the data points laid within the 1.96 standard deviations, as displayed with Bland Altman plots and agreement considered as strong. Hence, both methods could hypothetically be used for quantification, although CellProfiler has the benefit of reproducibility for both research and potentially care routines.

By developing these pipelines and validating them as a quantitative method, we showed a robust tool by which to perform multiple standardised outputs with increased transparency.

8.5 IMMUNOPATHOLOGICAL PROFILING

Immunolocalisation of CD4 T_h-cells and CD8 T_c-cells were the predominant cell type in oral mucosal and MSG biopsies (**papers III and IV**). This is in line with studies of OLP, and investigations in om- and sg-cGVHD [98, 200]. Aggregations of T-cells were found in close association with the oral mucosal epithelium, and within the ducts and acini. Both cell types were found migrating into the epithelial structures. CD68 macrophages were also localised close to the oral mucosa membrane, and occasionally found in the epithelium and deeper into the lamina propria. Macrophage involvement in OLP has been little studied and warrants further investigation [201]. Oral mucosal histopathological activity (GII, GIII and GIV) was found with significantly ($p \leq 0.001$) increased CD4, CD8 and CD68 compared to G0. Whereas in the MSG, CD68 was commonly observed but with considerably less frequency. MSG histopathological diagnostics (GIII-GIV and score 1-score 2) was significantly ($p < 0.05$) raised with CD4 and CD8 compared to G0-GI, and Score 0 respectively. Quantification of CD68 localisation was only found to be significant ($p < 0.01$) within the MSG biopsy of GIII-GIV compared to G0-GI, which supports the view that macrophages are part of the primary infiltrate and might be used to assist the histopathological investigation [144].

CD1a DCs were observed in the oral epithelium, and as sporadic migrating cells in the lamina propria; however, immunolocalisation was found to display a patient-dependent variation. The only mucosal histopathological grade that showed a significant ($p \leq 0.005$) increase in CD1a levels were GIII compared to G0. In the MSGs, infrequent CD1a cells were observed. Both oral mucosa and MSG showed minor localisation of infiltrating B-cells (CD19 and CD20), in line with the findings of others [143, 144, 202 127, 141, 142]. SS infiltrate is commonly described with CD4 predominance, but DCs, B-cells and macrophages are also part of the primary response [110]. It is interesting that macrophages, but even more specifically CD1a DCs, are strongly associated with SS-focused infiltrate and have been suggested to increase the specificity of the focus-score and histopathological evaluation [194, 203]. This raises an interesting question, whether the pathogenesis of SS differs compared to the immune profile reported in sg-GVHD?

8.5.1 Oral mucosal immunopathology

In **paper III,** we investigated the overall oral mucosal clinical immune profile. We included the 212 biopsies from **paper I**, but 42 patient samples were excluded due to histological exclusion post further sectioning. Additionally, three samples were excluded due to apparent concurrent pathology with heavy B-cell infiltrate (Table 3). Diagnostic om-cGVHD, distinctive om-cGVHD and oral HCT controls that were described with significantly increased histopathological grades, were also found with significant increases of CD4, CD8 and CD68 immunostaining compared to healthy (*Figure 4*, **paper III**). CD8 infiltrate was mostly elevated in diagnostic om-cGVHD (5-fold $p < 0.001$), whereas distinctive om-cGVHD showed

prominent CD4 (4-fold p<0.001) and CD68 (11-fold p<0.001). This is an interesting finding which needs to be considered in association with treatment and disease state. However, no significant changes were observed between diagnostic and distinctive histopathological severity or with the primary immune cells, we decided therefore to investigate distinctive and diagnostic om-cGVHD as one compiled group. In addition, CD1a was non-significantly changed in all of the above clinical groups compared to healthy.

Table 3. Oral mucosal biopsies clinical classification. Distribution of the initial 303 biopsies analysed post histological and clinical inclusion in relation to the oral mucosal status. Healthy oral mucosal biopsies were obtained prior HCT and post-HCT (oral HCT controls). Biopsies with definitive lichenoid lesions were considered diagnostic om-cGVHD, whereas distinctive om-cGVHD involved manifestations without typical lichenoid patterns, such as erythema, ulcerations, atrophy, and pseudomembranous. Numbers of biopsies are presented.

CLINICAL CLASSIFICATION	ORAL MUCOSAL		ORAL MUCOSAL & MSG ASSOCIATION	
	Histopathology (n=212)	Immunopathology (n=167)	Histopathology (n=81)	Immunopathology (n=48)
Prior HCT	26	17	10	4
Diagnostic om-cGVHD	78	65	25	16
Distinctive om-cGVHD	44	33	21	16
Oral HCT controls	49	37	22	12
Healthy	15	15	3	-

Clinical severity

To better understand the involvement of immune cell components, we characterised om-cGVHD into mild (n=71) or severe (n=27) according to the clinical ulcerative/erosive and extensive lesions, as described by others and supported by the NIH OMRS (Figure 5) [60, 142, 146]. By associating the clinical groups with histopathological diagnostic grades; G0-GI – "inactive", GII – "possible", and GIII-GIV – "likely" om-cGVHD, we decided to exclude biopsies (n=12) with clinically distinctive lesions and inactive pathology. Remaining diagnostic om-cGVHD (G0-GI, n=15) was considered inactive and only CD68 immunostaining was found with significance (3-fold $p<0.01$) (Figure 5). Localisation of CD4 was the most stable (3- to 6-fold) with increased levels in clinical groups defined with pathological diagnostics of GII, GIII-GIV (Figure 5). CD8 showed significant and elevated levels (8- to 10-fold) in patients with severe pathology (GIII-GIV), which was in line with previous reports for OLP and om-cGVHD [142, 180]. T_c-cells in lichenoid om-cGVHD have been characterised as CD8 T_c1-cells, but some CD4 were also described with T_h1-cell transcription [142]. T_h1-cells have been suggested to play a role in early phases of the disease but are not as elevated in cGVHD severity as CD8 and CD68 [138, 142]. In addition, CD68 immunolocalisation fluctuated across various clinical and pathological groups but showed association to cGVHD severity, as previously reported by others (7- to 19-fold) (Figure 5) [142, 144]. The role for CD68 might involve antigen presenting functions and the cells may exhibit a pro- or anti-inflammatory phenotype. The increase of CD68 localisation observed in the current study are supported by others, associated with clinical and pathological cGVHD severity [142]. Furthermore, diagnostic om-cGVHD with hyperkeratotic plaque-like lesions were considered as a separate group (late fibrotic cGVHD, n=3) due to the revised 2014 NIH clinical criteria [5]. In our cohort, the late fibrotic cGVHD were not significantly different from healthy (Figure 5). The assessment for clinical groups of om-cGVHD (mild, severe, inactive, fibrotic) highlights the importance of ensuring a concise clinical description in combination with pathological criteria, to facilitate comparable and improved outcomes from clinical trials [4].

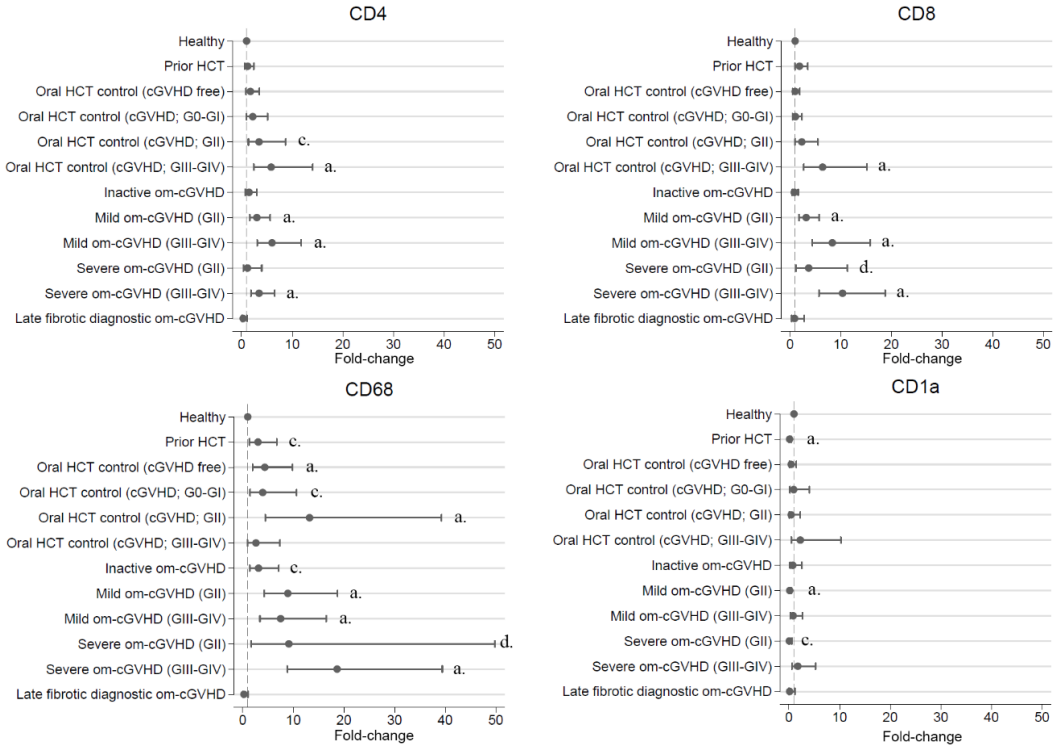

Figure 5. Immune profiles in clinical and pathologically defined om-cGVHD. Immune cell quantification of CD4, CD8, CD68 and CD1a presented as fold-change against healthy (dotted line, normalised as 1). om-cGVHD was investigated based on clinically mild, severe, and late fibrotic hyperkeratotic plaque. Diagnostic om-cGVHD with inactive pathology (G0-GI) was displayed as inactive om-cGVHD. The oral HCT controls were assessed as cGVHD free, or with cGVHD (G0-GIV). P values a=≤ 0.001, b=≤ 0.005, c=≤0.01 and d=≤ 0.05. *Figure from **paper III**: Tollemar., et al. Manuscript 2022.*

Acknowledging that oral HCT controls showed association with global cGVHD activity, we further analysed this group of patients based on overall cGVHD status (score 0-3). Oral HCT controls (cGVHD free, n=16) had an overall cGVHD score of 0 and were observed with a 4-fold increase in CD68 localisation ($p \leq 0.01$) but were non-significant for CD4 and CD8. Whereas oral HCT controls (with cGVHD, n=21) that had been diagnosed with non-oral cGVHD (scored 1-3) were found with wide pathological activity G0-GIV. The most interesting observations were that all oral HCT controls (cGVHD GIII-GIV) were obtained 0-3 months prior cGVHD onset and displayed a significant increase of CD4 and CD8 (6-fold $p<0.001$) but non-significant CD68.

cGVHD duration

In light of the above findings for oral HCT controls demonstrating subclinical cGVHD onset, we further assessed all om-cGVHD immune profiles associated to duration after HCT (*Table 2,* **paper III**). Biopsies were classified from initial cGVHD diagnosis into phases of onset (0-3 months), progression (3-6months), propagation (6-18months) and late phase (>18months) (Figure 5).

Above, CD68 has been described in association to oral HCT controls (cGVHD free), and inactive om-cGVHD (G0-GI). Analysed by duration, the inactive om-cGVHD (G0-GI) group presented with significant localisation of CD68 at onset (5-fold, $p<0.001$). Moreover, clinically mild and severe om-cGVHD also displayed significant CD68 localisation at onset (>7-fold, $p<0.005$) (Figure 6) (*Table 2,* **paper III**). In T-cell depleted grafts, there is evidence that macrophages restore conditioning-associated tissue damage without initiation of GVHD. Therefore, hypothetically host macrophages could have the capacity to limit GVHD, whereas donor macrophages could initiate GVHD [204-206]. LCs were found to be significantly reduced in om-cGVHD (GII) during onset and progression (≤8-fold, $p<0.05$), although at later time-points this group was not significant (*Table 2,* **paper III**). Professional APCs remain a subject for discussion and investigation within the field, with many conflicting hypotheses [207-210]. One such investigation, reported a population of CD68 cells to also express CD2ap for plasmacytoid DC phenotype [142]. This CD68+CD2ap+ population was associated with om-cGVHD severity [142]. Plasmacytoid DCs have been suggested to migrate into the oral mucosa upon inflammation, at the site secretion of type 1 IFN cytokines leading to a storm of chemokines and cytokines, and subsequent cGVHD initiation [142, 211].

The pattern of T-cell infiltration over the disease duration supported our previous findings. The CD4 population remained stable over time (3- to 5-fold, $p<0.05$) (*Table 2,* **paper III**)). However, at onset and progression, CD8 immunolocalisation was linked to all om-cGVHD patients with active pathology (>3-fold, $p<0.005$), with elevated levels in clinical and pathologically severe biopsies (>8-fold, $p<0.001$) (Figure 6). However, during late disease duration CD8 levels diminished, whereas the CD68 immune profile predominated and CD4 infiltrate remained with frequent levels (Figure 6). One study has reported an increased proportion of T_h-cells in relation to T_c-cells to occur later with om-cGVHD duration [99, 142]. Another study proposed that T_h-cells might become T_h2-polarised during om-cGVHD progression with erosive distinctive manifestations [99, 138].

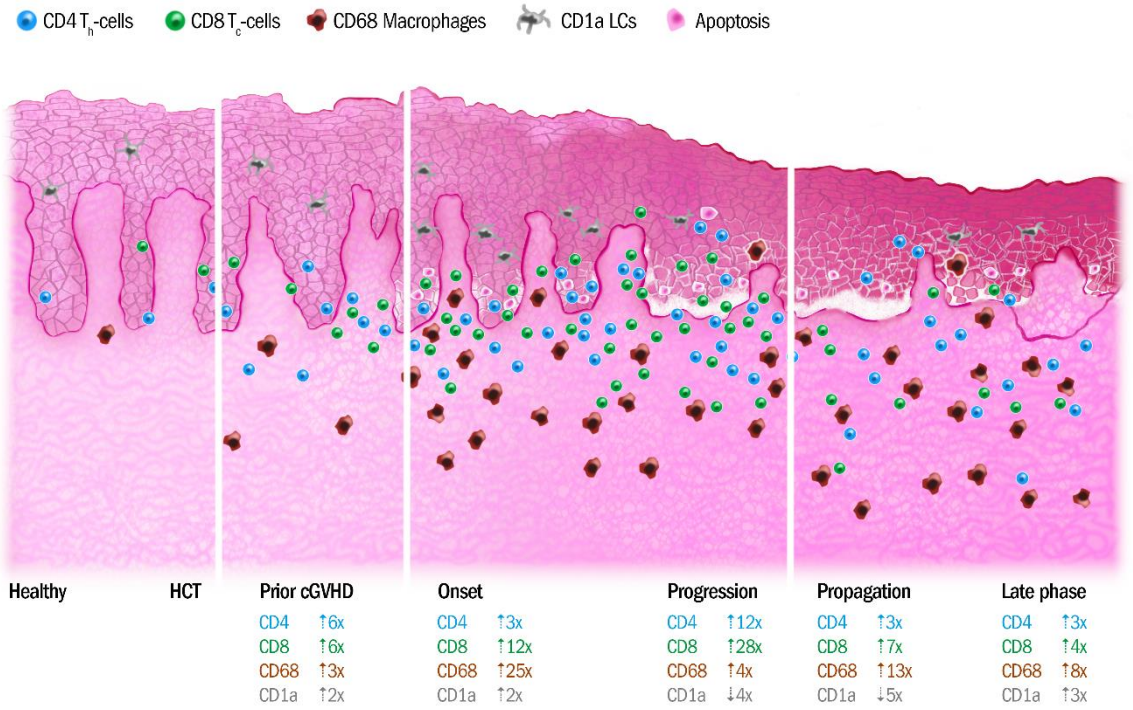

Figure 6. Immunopathological profile in severe om-cGVHD (GIII-GIV) duration. A schematic illustration of immunopathological changes in the oral mucosa following HCT and initiation of cGVHD. Histopathological features of apoptosis, liquefaction degeneration, basal membrane alterations and epithelial atrophy were observed during cGVHD onset, progression, propagation, and late phase. However, despite clinical and pathological characterisation of severe om-cGVHD (GIII-GIV) characterisation of immune cell levels and localisation displayed considerable variations. Numbers pertaining to the antibodies CD4 (blue), CD8 (green), CD68 (brown) and CD1a (grey) represent the fold-changes for severe om-cGVHD (GIII-GIV) against healthy. *Figure from **paper III**: Tollemar., et al. Manuscript 2022. Illustrator Mats Ceder.*

8.6 ORAL MUCOSAL AND SALIVARY GLAND ASSOCIATION

In **paper IV**, 78 HCT-biopsies were examined due to the presence of both oral mucosal and MSG tissue histology (Table 3). The biopsies had been assessed with the NIH cGVHD pathology score and diagnostic pathology grade. 48 HCT-biopsies had also been immunohistochemically stained for the CD4, CD8, CD68 and CD1a immune profile on both tissues, and were subsequently investigated for the association between om- and sg-cGVHD (Table 3). The research field has earlier reported a pathological agreement between mucous membrane and MSG histopathology; however, MSG pathological diagnosis have been suggested to predict the overall cGVHD activity more accurately [22, 186, 192, 202]. Thus, we investigated the association between the two tissues for the whole cohort and found a moderate pathology score correlation (r 0.40, $p<0.005$), as well as for infiltration of CD4 (r 0.69, $p<0.001$) and CD8 (r 0.51, $p<0.005$). In addition, we investigated any association with respect to disease duration. cGVHD onset revealed a strong and significant ($p<0.001$) correlation of pathology scores (r 0.73), CD8 infiltrate (r 0.84), and a moderate CD4 association (r 0.68, $p<0.01$). No correlation was found in pathology score and CD8 infiltrate as cGVHD progressed. Recent clinical data support that om- and sg-cGVHD are different pathophysiologies [18, 129] To our knowledge, our report is the first to explore the duration differences in the immunopathological profiles, indicating that om- and sg-cGVHD are different manifestations of oral cGVHD.

9 CONCLUSIONS

- Oral mucosal histopathology include lichenoid-like interface inflammation (exocytosis, apoptosis and liquefaction degeneration). In addition, basal membrane alterations were the most significant feature for diagnostic om-cGVHD.

- MSG histopathology can be assessed using the specific NIH cGVHD grading criteria, as well as the Greenspan composite score to evaluate disease activity.

- Pathological diagnoses of "inactive", "possible" and "likely" can be consistently assessed using our grading template.

- CD4 T_h-cells were found with stable levels over time in both the oral mucosa and MSG tissues, but mainly associated with mild distinctive manifestations.

- CD8 T_c-cells drives the histopathological damage, and were associated with diagnostic and severe clinical lesions, particularily during cGVHD onset and progression.

- CD68 macrophages were found in various clinical manifestations, but were associated with distinctive severe and late disease stages.

- At cGVHD onset, oral mucosal biopsies with affected or non-affected mucosa, including MSG segments, display immunopathological changes associated with overall cGVHD activity. As cGVHD progressed, little association between MSG and the oral mucosa was found.

10 POINTS OF PERSPECTIVE

In times of personalised medicine, the field of cGVHD need better tools to characterise patients with clinical activity or fibrosis, histopathological severity or aberrant tissue formation, with consideration of disease state [4]. Improved stratification would result in a more homogenous patient population, with the goal for pre-emptive and prognostic biomarkers [67]. Treatment strategies are also moving from broad and generalised immunosuppression to targeting disease specific pathways and manifestations [68].

Oral cGVHD is a heterogenous disorder affecting both oral mucosal and salivary gland tissue. om-cGVHD has been widely studied but with minimally defined criteria, in comparison to sg-cGVHD [5, 58]. Early clinical recognition of sg-cGVHD is complex, and a biopsy might only reflect systemic severity or earlier inflammation [58]. Therefore, saliva might be a useful source for identification or early biomarkers [212, 213].

CD4 T_h-cells and CD68 macrophages are plastic and with the ability to polarise into various functionalities warrant further investigation associated with disease state [99, 214, 215]. CD8 T_c-cells were found as the main driver for tissue destruction and diagnostic clinical severity but diminish with time, reflecting the transition from active inflammation into aberrant tissue remodelling [4, 99]. A knowledge gap remains for morbid forms of om-cGVHD, including the risk for cancer transformation [85]. Today, diagnosis and management rely on clinical surveillance, but might be accompanied with a biopsy showing active disease signs or dysplasia. Biomarkers in tissue would add support for clinical care, to understand which patients that have risk for morbid forms and cancer development.

In conclusion, cGVHD onset is linked to an elevated immunopathology in healthy and lesion mucosa, as well as in the salivary gland, suggesting systemic measurable activity. Evidence points out that progression of cGVHD continues with tissue specific pathways. Importantly, patients with mild, and severe clinicopathology, present with significantly different immune cell profiles due to the pathobiological differences during onset, progression, propagation, and late phase. Late phase of oral cGVHD with decreased mucosal integrity due to sg-cGVHD, and sclerotic mucosal features due to om-cGVHD need further investigations. Hence, improved clinical and pathological characterisation together with assessments in line with biological time-points are needed to improve the outcomes of future research. As a result, this will lead to an increased understanding of GVHD biology and improved patient stratification [67, 85].

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