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University of Turku

# HETEROGENEITY OF ORAL CAVITY CANCER WITH SPECIAL ATTENTION TO IMMUNE FUNCTION OF CLEVER-1

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## ABSTRACT

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### **Title: Heterogeneity of oral cavity cancer with special attention to immune function of Clever-1**

Various threats are fought off by human defense mechanisms each day. These threats may be foreign, such as microbes, or endogenous, such as cancer cells. Since cancer cells are essentially the host's own by origin, anti-cancer defense is a challenging task for the immune system.

This thesis work discusses the control of immunological responses, which may be dysregulated in the context of cancer, and the properties of cancer cells, which may determine their aggressiveness. This work focuses mainly on oral cavity squamous cell carcinoma. Special interest is shown to the Common Lymphatic Endothelial and Vascular Endothelial Receptor (Clever)-1, a scavenger receptor with multiple functions.

Clever-1 is expressed in the tumor microenvironment of various solid tumors. Its inhibition by monoclonal antibodies or genetic deletion may inhibit tumor growth in mice. The exact mechanism by which Clever-1 targeting inhibits cancer growth is still incompletely understood. As a potential therapeutic target in cancer, its significance in normal immune responses demands investigation.

The first part of this thesis focused on the function of the humoral immune response in Clever-1 deficient settings. The work revealed vigorous humoral responses in Clever-1 deficient mice, in particular towards polysaccharide type antigens. Accelerated antibody responses should not subject the individual to immune-mediated adverse events; they may even contribute to the anti-cancer effects of Clever-1 blocking therapies.

The second part of my thesis project shows that high risk patients may be identified by immunohistochemical biomarkers. Clever-1 expression in these tumors was not clearly associated with clinicopathological parameters. Of the studied prognostic biomarkers, analysis of CD44 and Hypoxia Inducible Factor 1 $\alpha$  allowed the identification of high risk patients, among unstratified patients with early stage OSCC.

In my work I discovered a new association between Clever-1 and humoral immunity. I also identified a potential way to prognosticate early stage OSCC patients.

**Keywords:** Clever-1, Biomarkers, Oral cavity squamous cell carcinoma, Humoral immunity, Immunology

## TIIVISTELMÄ

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### **Otsikko: Suuontelosityövän monimuotoisuus ja Clever-1:n merkitys puolustusmekanismeissa**

Immuunipuolustus torjuu monia uhkia. Nämä voivat olla peräisin ympäristöstä, kuten mikrobit, tai elimistöstä itsestään, kuten syöpäsolut. Syöpäsolujen torjunta on puolustusjärjestelmälle erityisen hankalaa, sillä ne ovat elimistön omia soluja.

Tässä väitöskirjassa käsittelen immuunipuolustuksen säätelyä, joka voi olla viallinen syöpää sairastavilla potilailla, sekä syöpäsolujen ominaisuuksia, jotka saattavat määrätä syöpäkasvaimen aggressiivisuuden. Keskityn erityisesti suuontelosityöpään. Käsittelen myös Common lymphatic endothelial and vascular endothelial receptor (Clever)-1 -nimistä reseptoria, joka toimii immuunipuolustuksessa monin tavoin.

Clever-1 ekspressoituu syöpäkasvaimissa monissa solutyypeissä, kuten verisuonissa ja valkosoluissa. Tuoreet tutkimukset ovat osoittaneet, että Clever-1:n toiminnan esto monoklonaalisilla vasta-aineilla tai geneettisellä manipulaatiolla voi hidastaa kasvainten kasvuja hiirillä. Sen toimintamekanismeja ei toistaiseksi täysin tunneta.

Clever-1 on mahdollinen syöpähoidon kohde, joten tutkin tässä väitöskirjassa sen vaikutusta normaaliin immuunipuolustuksen toimintaan. Tämä on tärkeää ottaa huomioon jotta mahdollisen Clever-1:een kohdistuvan hoidon haittavaikutuksia voidaan arvioida. Työssä käytin Clever-1 -poistogeenistä hiirtä, joka mahdollisti Clever-1:n vaikutuksen joustavan ja spesifisen tutkimisen. Tulokset osoittivat, että Clever-1:n puutos tehosti vasta-ainevälitteistä puolustusreaktiota. Poikkeuksellisen voimakkaan vasta-ainevälitteisen puolustusreaktion ei uskota altistavan puolustusjärjestelmän toimintaan liittyville haittavaikutuksille, vaan se voi jopa tehostaa syöpään kohdistuvaa puolustusvastetta.

Väitöskirjan muissa osajulkaisuissa totean, että korkean riskin suusyöpäpotilaat voidaan tunnistaa immunohistokemiallisten merkkiaineiden avulla. Näissä aineistoissa kasvaimissa tavattavan Clever-1:n määrä ei ollut yhteydessä ennusteeseen. Julkaisuissa raportoiduista ennusteellisista merkkiaineista CD44 ja Hypoxia Inducible Factor (HIF)-1 $\alpha$  yhdessä helpottivat korkean uusiutumisen riskin suuontelosityöpäpotilaiden tunnistamista varhain diagnosoitujen potilaiden joukosta.

Väitöstyössä tunnistin uuden yhteyden Clever-1:n ja humoraalisen puolustusvasteen välillä. Löysin myös mahdollisen tavan arvioida varhaisvaiheen suuontelosityövän ennustetta.

**Avainsanat:** Clever-1, Immunologia, Merkkiaine, Suuontelosityöpä, Vasta-aineet

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## ABBREVIATIONS

acLDL	Acetylated Low Density Lipoprotein	HNSCC	Head and Neck Squamous Cell Carcinoma
ADCC	Antibody Dependent Cellular Cytotoxicity	HPV	Human Papillomavirus
ADCP	Antibody Dependent Cellular Phagocytosis	HSC	Hematopoietic Stem Cell
APC	Antigen Presenting Cell	IL	Interleukin
BCG	Bacillus Calmette Guerin	ILC	Innate Lymphoid Cell
BCR	B Cell Receptor	mAb	Monoclonal antibody
BSA	Bovine Serum Albumin	MHC	Major Histocompatibility Complex
CAM	Cell Adhesion Molecule	MZB	Marginal Zone B cell
CD	Cluster of Differentiation	OSCC	Oral Cavity Squamous Cell Carcinoma
Cleaver-1	Common Lymphatic Endothelial and Vascular Endothelial Receptor 1	PD-(L)1	Programmed Death (Ligand)-1
CSC	Cancer Stem Cell	PET	Positron Emission Tomography
CT	Computed Tomography	S1P	Sphingosine 1 Phosphate
CTLA-4	Cytotoxic T Lymphocyte Antigen-4	SPARC	Secretory Protein, Acidic and Rich in Cystein
DFS	Disease Free Survival	TAM	Tumor Associated Macrophage
DSS	Disease Specific Survival	TLR	Toll-like Receptor
ECM	Extracellular Matrix	TM	Tumor Microenvironment
EGF(R)	Epidermal Growth Factor (Receptor)	TNF	Tumor Necrosis Factor
HEV	High Endothelial Venule	VEGF	Vascular Endothelial Growth Factor
HIF	Hypoxia Inducible Factor	WHO	World Health Organization

## **LIST OF ORIGINAL PUBLICATIONS**

This work is based on the original publications listed below, which are referred to by Roman numerals (I-III). The original publications have been reproduced with the permission of the copyright holders.

- I *Cleaver-1/Stabilin-1 Regulates Constitutive and Inducible Antibody Responses in Vivo*. Dunkel J, Karikoski M, Rantakari P, Hollmen M, Jalkanen S and Salmi M. Manuscript.
- II *Prognostic Markers in Stage I Oral Cavity Cancer*. Dunkel J, Vaittinen S, Grénman R, Kinnunen Ilpo and Irjala, H. *Laryngoscope*, 2013 Oct;123(10):2435-41.
- III *Tumoral expression of CD44 and HIF1 $\alpha$  predict stage 1 oral cavity squamous cell carcinoma outcome*. Dunkel J, Vaittinen S, Koivunen P, Laranne J, Mäkinen MJ, Tommola S and Irjala H. *Laryngoscope Investigative Otolaryngology* 2016 Feb;1(1):6-12.

## 1. INTRODUCTION

A functional immune system is an absolute prerequisite for higher forms of life. Its most important task is to make the distinction between 'self' and 'foreign'. 'Self' structures need to be tolerated, while 'foreign' structures have to be rejected<sup>1</sup>. Failure at either end will have dramatic consequences. When abnormal autoantigens or neoantigens, such as those found in cancer, are not sufficiently recognized by the immune system, tumors will be permitted to grow.

The normal lifespan of a malignant tumor may be divided into the following four stages: initial cancer-inducing mutagenesis, non-invasive growth *in situ*, locally invasive growth that breaches anatomical borders and metastatic spread via blood and lymphatic vessels. The kinetics of these events varies according to the tumor type, but these general principles apply to virtually all malignant tumors. At first tumors grow locally, but most of them develop a tendency to metastasize if not treated early enough.

The most common route that cancer cells use to metastasize is the lymphatic system. Such is also the case in head and neck cancer, where metastases are most frequently found in cervical lymph nodes. The progression of head and neck cancer is highly variable. It is currently unclear why some patients' tumors have a tendency to spread already at very early stages of the disease, whereas others may have locally advanced cancer along with healthy lymph nodes.<sup>2</sup>

Head and neck squamous cell carcinoma (HNSCC) appears to consist of various types of tumors. The human papillomavirus (HPV) has recently been identified as an important etiological factor in squamous cell carcinoma of the oropharynx, and HPV-associated oropharyngeal cancer has been shown to correlate with a more favorable outcome compared to those not associated with HPV<sup>3</sup>. Such associations are not seen in oral cavity squamous cell carcinoma (OSCC). In fact, none of the molecular features of oral cavity tumors are currently used to estimate cancer aggressiveness in clinical settings<sup>4</sup>.

Cancer targeting by immune-mediated therapeutics is currently under vigorous research and development. New immunoregulatory therapeutics such as ipilimumab (anti-cutaneous T lymphocyte antigen [CTLA]-4 mAb) and nivolumab (anti-programmed death [PD]-1 mAb) have been applied to head and neck cancer patients with promising results, but their effects appear to be limited to a subgroup of patients. Those who do not belong to this subgroup should all be treated by aggressive treatment regimens, including extensive surgical procedures and radiotherapy, but even these may be inadequate<sup>5</sup>. In addition to their insufficient effect, another drawback of these extensive therapies is a high rate of treatment-associated morbidity.

A potential novel target for cancer immunotherapy is the scavenger and leukocyte trafficking receptor Common Lymphatic Endothelial and Vascular Endothelial Receptor (Clever)-1. It is expressed in head and neck cancer, and its targeting has been shown to be effective in murine cancer models. Adverse effects of Clever-1 inhibition have not yet been extensively studied.<sup>6,7</sup>

In this work, the effect of Clever-1 silencing on the immune response was studied, with safety issues caused by its therapeutic inhibition in mind. Furthermore, suitable biomarkers that could estimate early stage oral cavity cancer severity were sought.

## 2. REVIEW OF THE LITERATURE

### 2.1. The immune system

The immune system consists crudely of immune cells and the immunological organs which they occupy. The migration routes used by immune cells, i.e. blood and lymphatic vasculature, are crucial. Most immune cells, or leukocytes, develop from hematopoietic stem cells in the bone marrow and are then released to protect the rest of the body through the peripheral circulation. Some leukocytes develop extramedullarily, i.e. independent of the bone marrow.

Adaptive immune cells, namely the lymphocytes, are first generated in the bone marrow. Next they are allotted their specific functions in the thymus or B cell follicles before their final activation in extramedullary secondary lymphoid organs, such as lymph nodes or spleen.

#### 2.1.1. Anatomy

Leukocytes develop in, populate, and travel through the primary and secondary lymphoid organs, and the blood and lymphatic vessels that connect them. In the periphery, leukocytes move out from the vessels, i.e. extravasate into tissues, where they search for invading pathogens. Secondary lymphoid organs, such as lymph nodes, spleen, tonsils and Peyer's patches are strategically located in sentinel positions so that the leukocytes they retain have the highest probability of encountering invading pathogens.

Lymph nodes develop in intersections of lymphatic vessels and the lymph draining from the periphery inevitably passes through them. The lymph contains interstitial fluid, leukocytes, and small particles that have entered the body, such as microbes. A part of the leukocytes that enter lymph nodes comes directly from the blood stream by extravasation from high endothelial venules (HEV). Since particularly the lymph nodes function as immunological sentinel organs, they are frequently the site of primary cancer metastasis.<sup>8</sup>

The spleen lies deep in the abdominal cavity and it filters the blood of blood-borne pathogens. As opposed to the lymph nodes, the spleen contains no afferent or efferent lymphatic vessels. Instead, all of the entering pathogens and leukocytes arrive and leave via blood vessels. Anatomically the spleen is comprised of the red pulp, most abundantly populated by tissue resident macrophages; the white pulp, most abundantly populated by lymphocytes; and the marginal zone in between these two contains a mixed population of macrophages, lymphocytes and accessory stromal cells. These different compartments participate in the clearance of aging blood cells, in adaptive immune responses, in extramedullary hematopoiesis, and several other crucial functions. In some circumstances, such as after trauma, the spleen has to be surgically removed. Splenectomized patients may live quite normal lives but their risk of microbial infections is substantially elevated.<sup>9,10</sup>

### **2.1.2. Development and key components**

Immune system development can be divided to the development of immune cells and stromal cells. However, these two are closely interconnected and interdependent.

Hematopoiesis, the formation of blood cells from hematopoietic stem cells (HSC), cannot take place without non-hematopoietic cells. The best described niche for hematopoiesis is the bone marrow. The bone marrow is the optimal site for HSC to develop, as there they are provided with diverse survival signals by the appropriate stromal cell populations<sup>11-13</sup>.

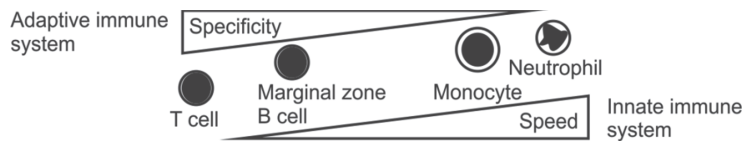
When leukocytes are released into the circulation, they face different fates depending on their lineage. Adaptive immune cells, in particular, need to continue their development in other lymphoid tissues, such as the thymus or B cell follicles. These migratory patterns are guided by leukocyte homing receptors, expressed on the surface of the leukocyte, and by their ligands on endothelial cells which are expressed in a tissue dependent manner on vascular endothelium. The tissue dependent expression of certain leukocyte trafficking receptors may be altered in tissue damage, and these alterations in the stromal cells may attract vast amounts of leukocytes to the site.<sup>14</sup>

Homing receptors may impart their function in various ways. They may function as chemokinetic receptors, which may attract the leukocyte to specific tissues, or as adhesins, which make the physical adhesion of the leukocyte to vessel walls possible<sup>15</sup>. Additionally, leukocytes may receive additional guidance from other receptor types, such as cell surface enzymes<sup>16</sup>.

#### **2.1.2.1. Leukocyte subsets**

Leukocytes may be crudely divided into innate or adaptive categories. In principal, innate leukocytes respond in a manner that is antigen unspecific and their response recurs at similar rates at each pathogen encounter. By contrast, adaptive leukocytes' responses are highly antigen specific and they are amplified after each pathogen encounter.

Abundant innate immune cells include granulocytes, monocytes, dendritic cells and macrophages. These cells respond to invading pathogens by releasing antibacterial substances or by engulfing and processing the pathogen. Classically the innate response has been described as one that develops very quickly and with the same intensity each time. In other words, the innate response is not associated with immunological memory. Conversely, the adaptive immune response is slower, but greatly specific (Figure 1).



**Figure 1.** The crude division between adaptive and innate immune cells, with four exemplary populations depicted. Most leukocyte subsets play roles in both innate and adaptive responses.

T and B lymphocytes are the best known adaptive immune cells. They leave the bone marrow in their immature state and complete their development in the thymus or in organized B cell follicles respectively. The mature T and B cells are highly proficient in the selective recognition of foreign macromolecules. Their high antigen recognition ability is explained by their ability to somatically edit their antigen receptors. The adaptive response is classically described by an initially slow response rate, which becomes faster, more specific and more robust after each antigen encounter, i.e. it develops a memory trace.

Functionally the B cells' responses are mediated by soluble antibodies that they produce. After activation via their B cell receptors, the B cells either develop into antibody secreting plasma cells (protective humoral immunity), or into memory B cells which can be quickly reactivated at the next antigen encounter (reactive humoral immunity). Secreted antibodies may be of structurally and functionally different isotypes, such as the pentameric IgM most commonly produced at the early phase after pathogen encounter, or the monomeric IgG produced at later phases.<sup>17</sup>

On the other hand, T cell responses are exerted locally by cell-cell contacts and targeted effector molecule secretion. They primarily respond by producing cytokines or cytotoxic effector molecules. The T cells recognize their antigens by specific T cell receptor ligation and co-stimulation provided by antigen-carrying professional antigen presenting cells (APC), which are leukocytes that specialize in harvesting pathogens to be displayed for other leukocyte populations.<sup>18</sup>

During the recent few decades, immunologists have become increasingly aware of the importance of cells in the middle of this classical division. Such cells include the innate lymphoid cells (ILC), which are leukocytes that lack myeloid features, resemble lymphocytes by appearance but are unable to somatically mutate their antigen recognition receptors<sup>19</sup>. Similarly to the ILCs, certain B cells have limited ability to edit their B cell antigen receptors (BCR)<sup>20</sup>. Such include the marginal zone B cells (MZB) and B1 cells. These B cell subsets respond rapidly when pathogens are encountered.

The MZB and B1 cells first develop in the bone marrow in the B cell lineage but, perhaps due to the specificity of their B cell antigen receptors, their fate is changed in the periphery. Perhaps the most important role of MZB and B1 cells is to provide immunity towards blood-borne pathogens<sup>21</sup>. They rapidly produce IgM class antibodies with broad specificity towards polysaccharides and lipids on the surface of bacteria,

and preferentially switch their isotype to IgG2a or IgG3 in mice<sup>22</sup>. MZB cells populate the marginal zone of the spleen, where they sample the blood for microbes. In mice the MZB cells are restricted to the spleen but in man, they are also found in the blood circulation and in tonsils<sup>23</sup>.

B1 cells are grossly functionally similar to MZB<sup>24</sup>. They reside in the spleen, but also in peritoneal and pleural cavities. Ontogenically B1 cells and MZB develop differently, and they have different preferences for isotype switching, where B1 cells typically switch to the IgA isotype<sup>25</sup>.

#### **2.1.2.2. Stroma**

Immunologists commonly refer to all but white blood cells as “stromal cells”. This terminology may depreciate the immunological role of stromal cells, as many of them play immunologically very active roles. For instance, stromal cells can provide or regulate chemokinetic and chemotactic cues for leukocytes<sup>26</sup>, they can mediate their adhesion and migration into tissues<sup>16</sup>, they can provide scaffolds where leukocytes can contact each other<sup>27</sup>, and they can present antigens and accessory signals for responding effector type leukocytes<sup>28</sup>. Stromal cells in lymphoid organs also have the ability to induce the differentiation of some leukocyte subsets<sup>29</sup>, and they can act as important promoters of specific immune responses<sup>30</sup>.

An important mechanism of immunological action by stromal cells is the production of the extracellular matrix (ECM). The ECM is composed of various macromolecules produced by mostly endothelial cells, epithelial cells and fibroblastic cells. These macromolecules are vital for the development of lymphoid organs and they are also crucial regulators of various immune functions, including leukocyte migration<sup>31</sup>. Structurally, ECM associated molecules can be subdivided into proteoglycans (e.g. heparan sulphate), non-proteoglycan polysaccharides (e.g. hyaluronate), fibers (e.g. collagens) and others (e.g. laminins).<sup>32</sup>

In terms of leukocyte trafficking, endothelial cells play the most crucial role of all stromal cells<sup>16</sup>. In addition to their role in direct leukocyte binding, they are also important producers of chemokines<sup>33</sup>. Chemokines produced by the endothelial cells are captured and presented by the proteoglycans on the endothelial cell surface, or on the ECM<sup>34</sup>.

#### **2.1.3. Control of the immune response**

The immune system comprises dozens of different leukocyte subsets, all with distinct features. The response of a single leukocyte depends on the activation signals it receives, and on the context in which they are delivered. The ultimate goal of the immune system is to produce a well-orchestrated response towards invading microbes, in order to protect the host from infections.

In health, the dynamic balance between insufficient and excessive leukocyte activation is maintained. When immune responses are insufficient, the individual is susceptible to infections by viruses, bacteria, fungi and parasites, as well as to cancer. When



immune responses are too vigorous, immune attack against healthy tissues becomes an issue. While the ways immune responses can be triggered have been made rather clear, the complex ensemble of immune control mechanisms still remains unraveled.

Accumulating knowledge on normal function of the immune system has opened new therapeutic possibilities. Immunological treatment modalities have been developed for both ends of the spectrum: autoimmunity and cancer. To date, targeting of the immune response in autoimmune disorders has been more widely implemented in the clinics. Drugs such as rituximab and etanercept, which target most activated B lymphocytes and the cytokine tumor necrosis factor (TNF) $\alpha$  respectively, have been revolutionary for millions of patients suffering from rheumatoid arthritis.<sup>35</sup>

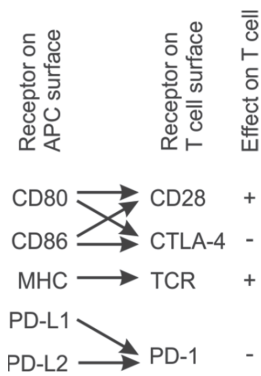
Nivolumab, a monoclonal antibody that targets the immune checkpoint marker Programmed Death (PD)-1 has been successfully tested in various tumors, including non-small-cell lung cancer and metastatic melanoma<sup>36</sup>. Other, more prototypic immunological treatments of cancer include adoptive T-cell transfer, which refers to the enrichment and delivery of the patient's own cancer-killing lymphocytes<sup>37</sup>.

As with all pharmacological treatments, drugs that manipulate immune function have their limitations. For example, PD-1 blocking therapy is most effective when tumors have upregulated PD-L1 as an immune escape mechanism<sup>36</sup>. In some cases, attempts to suppress immune activation have in fact enhanced immune responses. A dramatic example was the TGN1412 phase I clinical trial where the anti-CD28 mAb was expected to primarily influence regulatory T cells, but it in fact activated T-cells and caused a severe cytokine storm and multi organ failure in the participating healthy volunteers<sup>38</sup>.

#### **2.1.3.1. Immune control mechanisms**

Most immune interactions are controlled by several checkpoints. In order for leukocytes to commence with their effector functions, they need to receive primary stimulation (Signal 1), followed by co-stimulation (Signal 2), and possibly cues in the form of cytokines (Signal 3). The nature of these signals depends on the nature of the interaction, but generally it can be noted that lack of Signal 2 will lead to the unresponsive state of immunological tolerance.

In addition to co-stimulatory receptors and signals, leukocytes are also able to respond with anti-co-stimulatory or co-inhibitory signals from co-inhibitory receptors. Net effects of the most classical co-stimulatory and co-inhibitory receptors are depicted in Figure 2.



**Figure 2.** Classical receptors involved with antigen presentation, co-stimulation and co-inhibition in T cell activation. Major Histocompatibility Complex (MHC), Programmed Death receptor Ligand (PD-L)1/2, Cytotoxic T Lymphocyte Antigen (CTLA)-4, T Cell Receptor (TCR), Programmed Death receptor (PD)-1. + Stimulatory effect on T cell, - inhibitory effect on T cell.

The discoveries of specific co-inhibitory receptors and immunosuppressive leukocyte subsets, whose primary function is mediated by these receptors, have opened completely new horizons in immunotherapies. Cancer immunotherapies that exploit these forms of immune evasion are currently being extended to several new cancer types, particularly to those that contain a large mutational burden<sup>39,40</sup>.

#### 2.1.4. Immunological interactions

Virtually all immunological responses contain multiple interactions between several leukocyte types and stromal cells. As a relevant interaction in this work, splenic natural antibody production will be briefly described below.

Bacteria and other pathogens have access to the blood stream of all individuals every day. For protection from fulminant sepsis, protective levels of anti-bacterial antibodies are needed at all times (protective immunity). When the blood is breached at unusual rates, antibody levels need to be quickly elevated (reactive immunity).

While the conventional B cells produce large amounts of high affinity antibodies of different isotypes, their reaction time is too slow to fight bacteria that have already breached into the bloodstream. To overcome this issue, more “innate-type” B cell subsets such as MZB cells are needed. The MZB are specialized cells that produce antibodies without the help of T cells. MZB are constantly stimulated to produce antibody, even in infection-free conditions.

At least four known prerequisites are needed for the survival of MZB: (1) NOTCH2 signaling<sup>41</sup>, (2) integrin-mediated adhesion<sup>42</sup>, (3) positive selection through the B cell receptor (BCR) or toll-like receptors (TLR)<sup>43</sup> and (4) stromal orientation cues provided by chemokines and sphingosine-1-phosphate<sup>44,45</sup>. All of these aspects demonstrate the importance of the stromal cells in maintaining the normal function of leukocytes. The loss of any of these factors will result in dysregulation of MZB-driven humoral responses.

Blood borne pathogens are captured in the marginal zone by macrophages, and presented to MZB on the macrophages' scavenger receptors. This type of antigen presentation should not be confused with major histocompatibility complex (MHC)II

restricted antigen presentation since it takes place without endocytosis.<sup>46</sup> In the murine immune system, when an antigen is presented to the MZB, it rapidly starts to produce antibodies of the IgM and soon thereafter IgG3 isotypes<sup>21,47</sup>.

### **2.1.5. Inflammation**

Inflammation is classically defined as swelling, heat, redness and pain, combined with impaired normal function of the tissue. It is thought to have developed as a response to microbial infection. For immune response triggering, the responding leukocytes need to receive activation signals in a specific sequence. These signals can be referred to as immunological checkpoints, and they are crucial so that unwarranted activation, such as autoimmune inflammation, can be avoided.

Inflammation can be divided into acute or chronic forms. Important leukocyte subsets in acute inflammation comprise mostly innate leukocytes, such as monocytes and neutrophils. Chronic inflammation, on the other hand, is dominated by adaptive immune cells, such as lymphocytes. However, the division between these two is not clear-cut since many of their features and leukocyte subsets are shared.

#### **2.1.5.1. Leukocyte trafficking**

In order for inflammation to take place, leukocytes need to be able to reach their target tissue. This needs to be done in a coordinated manner since inflammation needs to be restricted to the appropriate site. Any other way would result in either an insufficient host response in the site where e.g. microbes have breached the body, or an unwarranted inflammatory damage to healthy tissues. As mentioned above, targeted leukocyte trafficking is mediated by chemoattractive receptors, adhesins and other supplementary molecules expressed by stromal cells in an induced manner.<sup>48</sup>

In addition to their functional division by response specificity, it might also be reasonable to divide leukocytes according to their migratory behavior. Some leukocyte subsets, such as dendritic cells, spend most of their time in tissues, where they guard their territory for invading pathogens. Others, such as lymphocytes, may continuously patrol the body in search for pathogens. A key difference between the leukocytes that sentinel at anatomical barrier tissues (such as the skin, gut and airways), and those that patrol the entire body, is antigen specificity. Typically sentinel type leukocytes such as dendritic cells may respond to a wide variety of pathogens, whereas patrolling lymphocytes may recognize only one specific antigenic epitope.

Patrolling lymphocytes need to keep watch over the entire body, with up to 100 000 km of blood vessels. Furthermore, lymphocytes typically carry only one specific antigen receptor out of hundreds of millions of different options, and a specific antigen receptor clone may be found in only a few hundred individual cells<sup>49</sup>. Combined, these two matters indicate that leukocyte trafficking needs to be a meticulously regulated process.

The encounter and phagocytosis of pathogens typically activates sentinel dendritic cells, and induces them to migrate. Migratory dendritic cells then start their journey towards the lymph nodes that drains the afferent lymph vessels from their region<sup>50</sup>.

These lymph nodes are the site where dendritic cells have the highest probability of encountering a lymphocyte with the correct specificity for the pathogens that particular dendritic cell carries. In order to travel from peripheral tissues to lymph nodes, dendritic cells need to express several key molecules including chemokine receptors and matrix metalloproteinases<sup>51</sup>. Many of these trafficking molecules expressed by leukocytes may also be expressed by metastasizing cancer cells<sup>52</sup>.

#### **2.1.5.2. Clinical significance of leukocyte trafficking with respect to cancer**

Cancer metastasis has three major ways of spreading: the hematogenous route, the lymphatic route and the route through bodily cavities such as the peritoneum<sup>53</sup>. The most common of these is the lymphatic route, which may be responsible for up to 80% of all metastases<sup>54</sup>.

Lymph node metastasis takes place via the afferent lymphatic vessels. The molecular mechanisms also overlap with those of physiological dendritic cell migration to lymph nodes. For instance, cancer cells may express the chemokine receptors CCR7 and CXCR4, which are among the most important chemokinetic molecules that guide leukocytes to lymph nodes<sup>55</sup>. As such mechanisms are overlapping between leukocytes and cancer cells, the study of mechanisms in leukocyte trafficking also often translates to cancer.

#### **2.1.5.3. Cancer associated inflammation**

Inflammation is one of the key features in the tumor microenvironment (TM)<sup>56</sup>. Cancer cells and leukocytes actively communicate and manipulate each other's behaviors<sup>57</sup>. During rapid tumor growth, large amounts of cancer cells also die in the process. This can take place under programmed conditions in apoptosis, by self-engulfment in autophagocytosis, or by uncontrolled means in tumor necrosis. Programmed cell death will generally lead to immunological tolerance, whereas uncontrolled cell death will elicit immune functions<sup>58</sup>.

Necrotic or immunogenic cancer cell death may release cytokines and chemokines, which attract tumor targeting leukocytes. On the other hand, these cytokines may also promote cancer cell growth<sup>59</sup> and metastatic processes. While chemokines are initially produced to improve immunity, they might also play a role in metastasis development<sup>60</sup>.

For efficient anti-tumor responses, lymphocytes and key cytokines such as interferon gamma are needed<sup>61</sup>. Lymphocytes are able to target tumors when they recognize foreign (mutated or viral) epitopes, or epitopes expressed at significantly higher levels than in healthy tissues. From genetically manipulated mouse models and immunocompromised patients, we know that the tumors that grow large enough to be detectable are the ones that have escaped immune recognition. In other words, they have undergone immunological selection. Cancer that has arisen in immune deficient settings is immunologically unselected, and would be easily eliminated by an intact immune system<sup>62,63</sup>.

Tumors caused by viral infections can potentially be more immunogenic than those caused e.g. by mutations in signaling pathways. This is apparent in head and neck cancer caused by HPV infection, which may be immunohistochemically identified by their expression of p16<sup>INK4a</sup>, among other methods. Intriguingly, the survival advantage of head and neck cancer patients with HPV positive tumors is seen only when the HPV positive tumors contain large amounts of cytotoxic T cells<sup>64</sup>. This may imply that cancer-targeting T cells are concentrated in tumors with recognizable foreign (viral) epitopes, and they exert their effector functions most effectively in this case. Alternatively, the survival benefit of HPV positive head and neck tumors may be dependent on genetic features. A recent report has described a survival advantage in patients with mutations in the Interleukin (IL)-10 encoding gene<sup>65</sup>. IL-10 is an important defense mechanism against HPV infection, but it also has several anti-inflammatory roles in the immune system. Thus, patients harboring this particular mutation are more susceptible for the infection, but more resistant against the cancer.

For the cancer patient, inflammation may be either beneficial or harmful. On the one hand, cytotoxic T cells and phagocytes are recruited to kill cancer cells. On the other hand, cancer cells may in some cases selectively attract certain leukocyte subsets, such as regulatory T cells, to suppress anti-cancer responses. Recent evidence suggests that tumors may change the phenotype of tumor associated vasculature to make only immunosuppressive leukocytes able to enter the tumor stroma<sup>66</sup>. In this study by Motz and colleagues, cancer cells produced leukotrienes to upregulate the pro-apoptotic molecule FasL on endothelial cells, which in turn gave regulatory T cells an advantage over other T cell subsets in reaching the tumor. This example, along with the activation of the PD-1-PD-L1 immune checkpoint axis described above, demonstrates that cancer cells develop ways to actively evade immune attack.

The immune microenvironment may also affect, and be affected by tumor associated macrophages (TAM). The TAMs often represent the most abundant tumor associated leukocyte population. They may be polarized by the TM to completely opposite directions, namely M1 and M2. M1 polarized TAM are pro-inflammatory by function, and mediate anti-tumor effects, whereas M2 polarized TAM may promote tumor survival. Generally, TAM appear to promote the progression of established tumors.<sup>67</sup>

### **2.1.6. Therapeutic targeting of the immune system**

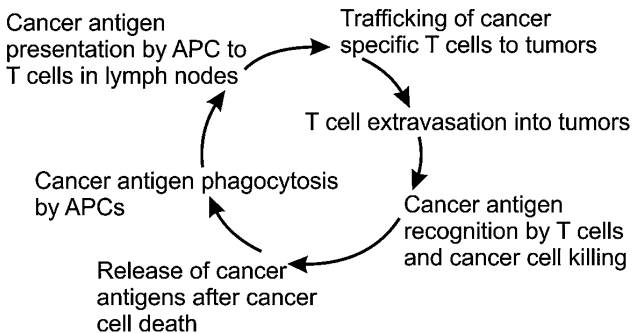
The immune system can be targeted in several ways and in numerous conditions, but only very seldom without adverse effects. Cancer and autoimmune disorders are at extreme ends of the immunotherapy spectrum, but the immune system may be targeted also in other conditions. These may include non-autoimmune inflammatory conditions, such as asthma, or the inhibition of rejection after organ transplantation. This review will focus on cancer immunotherapies.

#### **2.1.6.1. Cancer immunotherapy**

While treatment of autoimmune conditions by dampening of immune responses is, at least in theory, rather straightforward, boosting the immune system to better recognize

tumor cells may be more complicated. The major challenge is to confine therapeutic immune stimulation to the relevant sites, in order to avoid unwarranted damage to normal tissues. For these reasons, immunotherapies for autoimmune disorders are much more advanced and more widely implemented in clinics, than are those for cancer.

The tumor-immune system interaction has several important steps, as described in Figure 3. Cancer cells may try to disturb or interrupt these events in their attempts to survive. Such attempts, in turn, may in some cases be targeted by immunotherapy.



**Figure 3.** The most important events in the anti-tumor immune response, adapted from Chen et al <sup>236</sup>.

Since specific means of stimulating the adaptive immune system have only been established during the past 15 years, anti-tumor drugs that ubiquitously stimulate the innate immune system are the only ones widely established in oncological treatment today. In a way it can be said that cancer-targeting immunotherapies' spectra are becoming narrower.

Since the late 1970s, bladder cancer patients have been treated with *Bacillus Calmette Guerin* (BCG), a substance comprised of weakened mycobacteria, and which is more commonly used as a vaccine for tuberculosis<sup>68</sup>. Even though such ubiquitous immune stimulation has proven to be effective, its exact mechanism of action still remains uncertain. Several inflammatory mediators and leukocytes contribute to the anti-cancer efficiency of BCG treatment <sup>69–73</sup>, in addition to the proposed direct cytotoxicity of BCG on cancer cells<sup>74</sup>.

Another success in cancer immunotherapy has been the use of TLR agonists in various types of skin cancer. The TLR7 agonist imiquimod stimulates anti-tumor effector functions of several leukocyte subsets when applied topically, but not systemically<sup>75–77</sup>.

Activation of the innate immune system has proven to be particularly effective in cases, where the tumor targeted inflammation can be anatomically confined, such as in bladder cancer or skin cancer. However, ubiquitous boosting of the immune system can be more cumbersome when the tumor is not as well confined anatomically, or when it is harder to reach. This might be explained by the dilution of immune cells and inflammatory mediators throughout the body. Immunotherapy of metastasized tumors

in particular may not be plausible by systemic stimulation of the innate immune system.

Most cancer drugs that target the immune system improve the ability of T cells to recognize tumor cells. Therapeutic success is defined as a positive feedback loop with propagation and amplification of cancer cell recognition and killing by the immune system. Recently, cancer researchers' interests have been focused on immune checkpoint blockade. This refers to the pharmacological inhibition of Signal 2, which has been described above. Current clinical attempts focus mostly on CTLA-4 blockade or PD-1 blockade by monoclonal antibodies, such as ipilimumab<sup>78</sup> or nivolumab<sup>79</sup>, respectively.

Cancer geneticists have recently recognized that mutation frequencies of cancer cells vary tremendously according to the cell type and etiology of the tumor. Consequently, tumors with large mutational burden harbor several tumor associated neoantigens, any of which may potentially be recognizable by T cells as 'non-self'. Tumors with large mutational burden include melanoma, lung cancer and head and neck cancer. Tumors with low mutation frequencies include certain sarcomas and hematopoietic malignancies<sup>39</sup>. Particularly therapeutic targeting of the adaptive immune system in tumors with few mutations may be problematic, as recognizable non-self antigen receptor epitopes are scarce.

Antibodies that directly target cancer cells, and exert their functions via antibody dependent cellular cytotoxicity, may also be classified as immunotherapy. Such therapies exist for both hematopoietic as well as solid tumors. Among several potential objects, the most widely targeted cancer associated antigens include HER2, epidermal growth factor receptor (EGFR) and CD20.<sup>80</sup>

The enormous efforts of the cancer immunology society have yielded promising tumor targeting immunotherapies. Despite the established and promising anti-tumor effects of these drugs, they are by any means not perfect in many cases. For these reasons suitable novel pathways should be explored.

#### **2.1.7. Clever-1**

Clever-1 is a scavenger receptor and leukocyte trafficking molecule found mainly on endothelial cells and a subset of immunosuppressive macrophages. It is commonly also referred to by its alternative names FEEL-1 and Stabilin-1, and by its gene name *Stab1*. Together with Stabilin-2 (*Stab2*), also called HARE, they compose the Stabilin-group of scavenger receptors. These two receptors are 55% homologous at the protein level<sup>81</sup>.

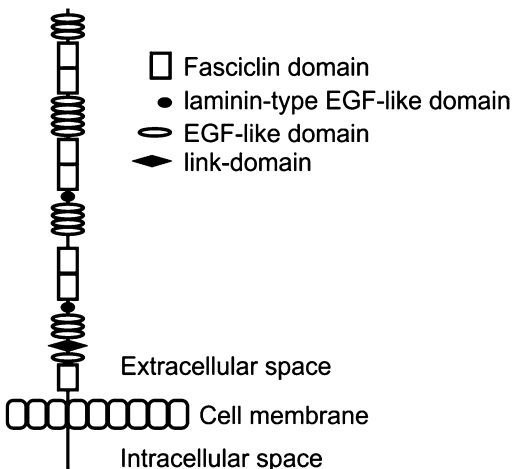
The MS-1 antibody, a monoclonal antibody raised against human spleen, was first described in 1991<sup>82</sup>. MS-1 identified sinusoidal non-continuous endothelium in various organs. The target of the MS-1 antibody was independently recognized by three different groups in 2002 as the Clever-1/FEEL-1/Stabilin-1 protein<sup>81,83,84</sup>.

Clever-1 was characterized as an approximately 280 kDa cell surface receptor with specificity for various ligands. Clever-1 expression was found mostly on endothelial cells and certain leukocytes of the myeloid lineage, and it was inducible by IL-4 and dexamethasone<sup>81</sup>.

The importance of Clever-1 in cancer growth has later been demonstrated in mice<sup>6,85</sup>. Clever-1 expression has been detected in the TM cells in various epithelial tumors, including breast cancer, head and neck cancer and colorectal cancer, but not in the tumor cells as such<sup>86,87</sup>. Its prognostic role in OSCC has not been previously studied.

#### 2.1.7.1. Structure and function of Clever-1

The human form of Clever-1 is 82% homologous to mouse Clever-1 at the amino acid level<sup>81</sup>. It contains 7 fasciclin (Fas-1), 16 epidermal growth factor (EGF)-like, 2 laminin-type EGF-like domains and a C-type lectin-like link module<sup>88</sup>. The first report describing its structure predicted its expression to be equally distributed between the Golgi, endoplasmic reticulum and plasma membrane, which implied rapid trafficking between these compartments<sup>81</sup>. Later, these results were confirmed in a paper by an independent group, where Clever-1 was seen to be mostly located inside the Clever-1 expressing cells, but it co-localized with the early endosome marker EEA1<sup>89</sup>. Clever-1 is attached to the cell membrane by a transmembrane domain and it contains only a short cytoplasmic domain (Figure 4).



**Figure 4.** Structure of Clever-1, adapted from Adachi et al <sup>88</sup>.

Clever-1 is functionally classified as a scavenger receptor. Scavenger receptors are a group of receptors that lack structural similarity but share specificity for various ligands<sup>90</sup>. They are responsible for the removal of damaged or unwanted “waste” molecules, which would otherwise accumulate in the body and cause problems. Such include acetylated low density lipoprotein (acLDL), tissue plasminogen activator and immune complexes, among many others.



The stabilin family receptors have both unique and shared ligands. Clever-1 and Stab2 both have the ability to scavenge acetylated LDL, advanced glycation end products<sup>83</sup>, GDF-15<sup>91</sup>, heparin<sup>92</sup> and bacteria<sup>88</sup>. Both receptors have additionally been proposed to mediate the clearance of cell corpses by the recognition and endocytosis of phosphatidylserine on the target cells<sup>93</sup>. Identified unique ligands for Clever-1 include Secretory Protein, Acidic and Rich in Cystein (SPARC)<sup>94</sup>, and placental lactogen<sup>95</sup>. Capacity for the clearance of hyaluronate, which has been proposed to be the main function of Stab2, is lacking from Clever-1<sup>89</sup>.

Experiments on cultured primary monocytes have suggested monocyte/macrophage restricted Clever-1 to be immune suppressive in function<sup>96</sup>. In these experiments, Clever-1 inhibition in antigen presenting cells ameliorates their ability to induce T cell activation.

In addition to the role of Clever-1 in scavenging and immune suppression, evidence on its role in leukocyte trafficking is emerging. In vitro binding assays have suggested that blocking Clever-1 with monoclonal antibodies can potentially inhibit leukocyte binding to both lymphatic and blood vascular endothelium on tissue sections<sup>84</sup>. A similar interaction has been shown in human umbilical vessel endothelial cells and liver sinusoidal endothelial cells by capillary flow assays<sup>97-99</sup>. Specifically, monoclonal antibodies that block Clever-1 impair the transmigration phase of the leukocyte extravasation cascade. Finally, in vivo animal studies have demonstrated the importance of Clever-1 in leukocyte trafficking from peripheral tissues to draining lymph nodes<sup>100</sup>. A potential Clever-1 ligand expressed by leukocytes, that would mediate their adhesion to the endothelium, has not yet been identified.

#### **2.1.7.2. Expression patterns and disease associations of Clever-1**

Clever-1 is most abundantly expressed by non-continuous endothelium. In fact, the MS-1 antigen, which was later identified as Clever-1, was used as an immunohistochemical marker for sinusoidal endothelium<sup>82</sup>. It was soon also noted that Clever-1 expression can be induced in continuous endothelium and monocytes by IL-4 and glucocorticoids<sup>101</sup>.

##### *2.1.7.2.1. Non-induced expression*

In health, Clever-1 is expressed predominantly by sinusoidal endothelial cells in the spleen, liver and bone marrow, by lymph node and tonsillar HEVs, and by peripheral lymphatic vessels<sup>82</sup>. It should be noted that expression patterns differ slightly between mouse and man, as Clever-1 expression is missing in murine HEVs<sup>100</sup>.

##### *2.1.7.2.2. Expression in cancer*

Lymphatic vessels express Clever-1, but in pathologic conditions Clever-1 expression is induced also on blood capillaries<sup>97</sup>. Since interstitial leukocytes readily cross the lymphatic endothelium, but the blood vascular endothelium only in very select situations, this phenomenon was soon recognized to be suggestive of the leukocyte trafficking function of Clever-1. Clever-1 expression has been detected in TAMs and lymphatic vessels in several cancer types<sup>86,102,103</sup>.

Associations between Clever-1 expression and disease course have been studied in clinical settings with material from bladder cancer<sup>104</sup>, colon cancer<sup>87</sup> and breast cancer<sup>105</sup> patients. In these studies, correlations between Clever-1 expression and clinical outcome were not completely consistent. Instead, the results from these studies indicate that Clever-1 expression levels in the tumor stroma change according to disease progression. It is notable, that Clever-1 expression is in practice not induced in diseases with strong associations to Th1 cytokines<sup>102</sup>.

## **2.2. Oral cavity squamous cell carcinoma**

Cancer is a heterogeneous group of diseases that are characterized by the uncontrolled growth of cells after undesirable mutations. While mutations of gametes are crucial for evolution and diversity, mutations of somatic cells are desirable only in rare selected cases. Somatic mutated cancer cells displace normal cells, spread to distant organs and ultimately, lead to the death of the individual. Mutation rates are known to vary greatly across cancer types, in a manner where those tumors often associated with environmental factors, such as radiation or chemicals, contain the largest amounts of mutations<sup>39</sup>.

Cancer cells dynamically interact with normal cells in their endeavors to grow and spread. The cancer cell niche, or TM, has attracted the interest of cancer researchers in the recent years. In the TM, leukocytes predestined to destroy the tumor can in fact be reprogrammed by tumor cells to promote their survival.

Tumors have traditionally been classified according to their anatomical localization and cell-type of origin. Common mutations in selected cancer types have been identified, such as BRAF mutations in melanoma and HER2 mutations in breast cancer. Specific mutations have proven to be suitable prognostic markers, as well as therapeutic targets. However, canonical mutations only account for select cases.

Malignant tumors of the oral cavity are most often derived from squamous epithelial cells. Even though all cases of OSCC are caused by mutations of the same cell type, disease courses are still highly variable among patients. OSCC can potentially be identified at early stages but still only one half of the patients remain in remission after treatment.

The most important prognostic feature in oral cavity cancer is the involvement of cervical lymph nodes at the time of diagnosis<sup>106</sup>. This is taken into consideration in the TNM classification published by the World Health Organization (WHO), where T represents the tumor's size and invasive properties, N the number and size of affected regional lymph nodes, and M distant metastases<sup>107</sup>. The scores from each category are summarized into stage, which is used in clinical decision making.

Stage 1 tumors are by definition under 20 mm in diameter (T1) and local (N0 and M0). Currently these patients are treated with a combination of tumor ablative surgery and sentinel lymph node biopsy, whereas chemotherapeutic and radiotherapeutic adjuvant treatment is typically reserved for more advanced stage tumors. Extensive treatment

would improve cancer specific survival at the population level, but the drawback is treatment associated morbidity and a deterioration in the quality of life of individual patients. Recurrent cancer is observed in about 30% of stage 1 OSCC patients.<sup>108</sup>

The treatment protocols of OSCC patients could potentially be supplemented with immune therapy. The advantages of immune therapy include precision and extensiveness, where appropriately programmed leukocytes are able to find and kill even cancer cells that have spread from their original site. However, the possible adverse effects of immunological drugs need to be extensively studied as they can become chronic and in some extreme cases, acutely lethal.

### **2.2.1. General features of cancer**

Cancer hallmarks have been extensively reviewed by Hanahan and Weinberg<sup>56</sup>. Key hallmarks include replicative immortality, genomic instability, self-sustained proliferative signaling, evasion of growth suppressive signals, resistance to cell death, angiogenesis, invasion and metastatic capacity, metabolic reprogramming, cancer promoting inflammation, and immune evasion. These features give cancer cells a survival advantage over normal cells, but they may also be used as targets in cancer therapy. In fact, cancer drugs have been developed against all of these hallmarks, with varying success.

Different tumors may be driven by unique cancer features, which vary among cancer types. The contribution of these key features may be identified by specific biomarkers, such as Ki67 in the determination of proliferative capacity. Generally, malignant tumors initiating from the same cell type may be driven by similar “hallmark profiles”, but heterogeneity also exists within the cancer types. Thus, emphasis should be given to the cancer specific molecular features, so that each patient’s therapy can be planned in a personalized and optimized manner.

The cancer cell niche comprehends all of the non-cancerous cells, extracellular matrix components, and growth factors that are present in or around the tumor. This niche supports tumor growth.

The immune system plays a multifaceted role in cancer progression. At first, responding leukocyte subsets directly phagocytose cancer cells, limit their growth by means of cytokines, and attract more leukocytes to attack them. When some cancer cells are able to resist this attack, a temporary state of equilibrium may be found. Once cancer cells have managed to swerve the initial immune attack executed by innate immune cells and pre-existing immunological memory, they need to start fine tuning their defense mechanisms against the developing new adaptive immune responses. The cancer cells may find various ways to actively escape leukocyte attack. This process resembles Darwinian selection at a small scale, and it can be referred to by the term ‘immunoediting’.<sup>109</sup>

### **2.2.2. Development of metastases**

In order to metastasize, epithelial in situ tumors need to gain the capacity to breach the basal membrane. For this to be possible cancer cells must disrupt the ECM, which is also a crucial element in cancer promotion<sup>110,111</sup>. In epithelial tumors this process defines invasiveness, and key effector molecules in this process include the MMPs<sup>112</sup>. After the basal membrane has been breached, cancer cells depend on the same migratory cues and routes that leukocytes use in order to reach peripheral tissues. Tumors can also improve their access to lymphatic vessels by promoting lymphangiogenesis, the generation of new lymphatic vessels<sup>54</sup>. A notable exception in metastatic processes is made by hematopoietic malignancies, where the cancer cells merely spread due to their intrinsic predispositions for homing<sup>113</sup>.

Each cancer type has typical sites where they preferentially metastasize. These sites, or pre-metastatic niches, are often remotely prepared by the tumors before metastasis takes place<sup>114</sup>. Correlations between tumoral expression of chemoattractive receptors and the tumors' metastatic potential have been documented in various cancer types<sup>115–117</sup>.

During the metastatic process, cancer cells are particularly vulnerable to immune attack. However, they may communicate with leukocytes, such as tumor associated macrophages, in order to reduce their anti-tumor response. The leukocytes educated by tumor cells may then communicate with tumor-engaging cytotoxic cells, and induce immunosuppression in them<sup>118</sup>. Communication between tumor cells and leukocytes are typically studied in co-culture assays, but the exact *in vivo* signals functioning in this process are incompletely understood.

Also, cancer cells may render the tumor microenvironment immunosuppressive in function. One such example is the induction of the death receptor ligand FasL on vascular endothelial cells, which induces apoptotic cell death of tumor homing cytotoxic T cells<sup>66</sup>.

### **2.2.3. characteristics of oral cavity malignancies**

OSCC is the most abundant malignancy in the oral cavity. Despite its close anatomical positioning it is not to be bundled together with squamous cell tumors of the oropharynx, as these two diseases have several unique features.

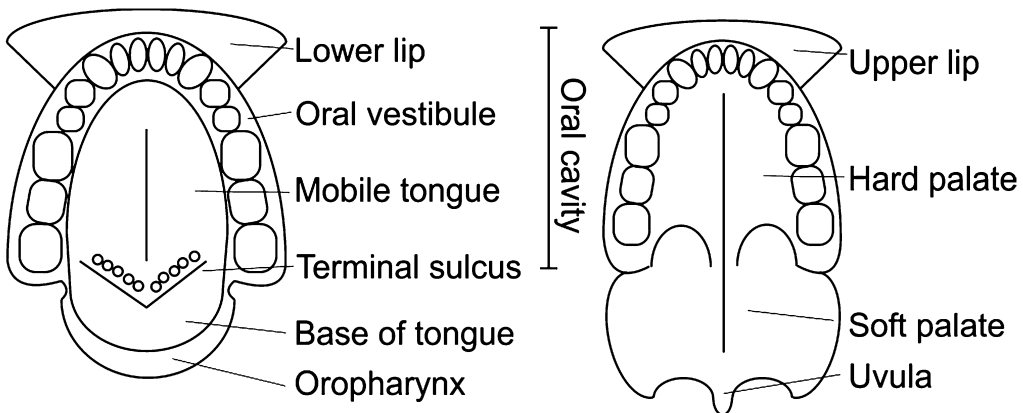
The currently studied aspects in the head and neck cancer field are outlined in Figure 5, and emphasized according to the subjective view of the author.



**Figure 5.** The hallmarks of cancer. A subjective view of the head and neck cancer research field's current emphasis to the different classical cancer hallmarks. Image inspired by Hanahan and Weinberg <sup>56</sup>.

**2.2.3.1. Epidemiology and classification**

Globally, 300 000 people are affected by OSCC each year<sup>119</sup>. Predisposing etiological factors may vary across cultures, but the population adjusted incidence rate of OSCC in the Finnish population is similar to the global average<sup>120</sup>. Data from the Nordcan database shows that age adjusted incidence has been steadily rising in Finland for as long as data has been recorded<sup>120</sup>. Currently OSCC is classified in the ICD-10 system according to its anatomical site and cell type of origin with the codes C00, C02-04, C05.0, and C06. These codes include malignant squamocellular tumors of the mobile tongue, gingiva, mouth floor, hard palate or undefined regions of the oral cavity respectively (Figure 6).



**Figure 6.** A schematic illustration of oral anatomy and related terminology.

Predisposing factors for OSCC include tobacco smoking, alcohol consumption, chronic irritation e.g. from dental prostheses and Betel quid chewing. The accumulation of risk factors increases the risk for OSCC in a multiplicative rather than an additive manner. Patients are more commonly males.<sup>121</sup> Even though smoking has become more uncommon for several decades in westernized countries, the age-adjusted incidence

of OSCC has remained the same for both men and women<sup>122</sup>, but trends in incidence vary across countries. As opposed to oropharyngeal cancer, a closely related malignancy, human papillomavirus infection is not considered to be an important predisposing factor in OSCC<sup>121</sup>.

#### **2.2.3.2. Anatomical considerations in OSCC**

Compared to tumors affecting deep tissues such as the internal organs, OSCC tumors are often visible and are thus subject to early detection. For this reason, OSCC patients may be at a theoretical advantage over those with tumors in most other sites. In the Finnish population about half of the patients with OSCC are diagnosed at early stages.<sup>123</sup>

Therapeutic interventions usually cause severe functional and cosmetic deficiencies for the patients. This is an important matter to consider when therapy is planned. Conversely, cancer recurrence in the oral cavity has dramatic consequences for the patient, which means that highly accurate prognostic tools are needed.

A considerable challenge to the interpretation of several HNSCC studies is that particularly in smaller studies tumors from several anatomical locations have been grouped together and analyzed in bulk. This matter may mask phenomena present only in select sites, such as the oral cavity. Thus, conclusions derived from these studies should be read with this in mind.

#### **2.2.3.3. Prognosis of OSCC**

The five year disease specific and disease free survival rates in locally restricted oral cavity cancer are about 85% and 70% respectively<sup>124</sup>. The main prognostic indicator in OSCC is currently tumor staging, which is based on macroscopic, radiographic and microscopic parameters determined by the clinician, the radiologist and the pathologist. Additional prognostic parameters may include tumor invasion depth, worst pattern of invasion, lymph invasion, perineural invasion, cancer cell differentiation, surgical margins and extracapsular spread<sup>125</sup>. Surprisingly, pre-treatment smoking habits had virtually no impact on treatment outcome in a large study with clinical data from several cultures<sup>126</sup>.

The presence and location of lymph node metastasis is one of the most important prognosticators for poor overall survival in OSCC<sup>127</sup>. Preoperative ultrasonography has been shown to be useful predictors of neck metastases by Meyer and colleagues, but their results also showed that ultrasonography alone may not be reliable enough to rule out neck metastases<sup>128</sup>.

A histological risk score to estimate the prognosis of early stage OSCC has been developed by Brandwein-Gensler and colleagues<sup>129,130</sup>. This method takes into account the worst pattern of invasion, tumor associated lymphocytes and perineural invasion. Unfortunately these findings were not reproducible in an independent study by Rodrigues and colleagues<sup>131</sup>.

An alternative scoring system has been proposed by Almgush and colleagues<sup>132</sup>. Their model appears rather straightforward as it takes two well described histological features into account: tumor budding and depth of invasion. A validation study of this model in a prospective study would be highly interesting.

#### **2.2.3.4. Cellular and molecular features of OSCC**

Head and neck cancer, including OSCC, is the culmination of precancerous stages. A key feature in this process is epithelial-mesenchymal transition, where epithelial cells lose their adhesive properties and polarity, and become migratory mesenchymal stem cells. At a cellular level, OSCC may develop in areas with aberrant precancerous mutations, a process also known as field cancerization, where theoretically any of the premalignant cells may develop to cancerous stages<sup>133</sup>. Since fields often exceed surgical margins, secondary tumors of different clonality to the primary tumor often develop in these patients. Macroscopically, the changes associated with field cancerization may in selected cases be seen as leukoplakia in the oral cavity. Malignant transformation of leukoplakia to cancer occurs at an annual rate of 1-2%<sup>134</sup>.

Cancer cells accumulate the ability to maintain a replicative cell cycle. Control of the cell cycle is complex, but the most common pathways affected in head and neck cancer are related to the tumor suppressor proteins *p53* and retinoblastoma (*pRb*). These proteins are important inhibitors of cell cycle progression. Special interest has been shown towards these proteins since the association between HPV infection and *p53* deletion was reported<sup>135</sup>. *p53*-mediated cell cycle arrest can be inhibited by somatic mutations in *p53*<sup>136</sup> or by HPV-mediated silencing of *p53*<sup>137</sup>. Of head and neck tumors, those in the oropharynx are usually associated with HPV, while oral cavity tumors are more commonly associated with *p53* mutations<sup>138</sup>. Similarly to *p53*, the tumor suppressor *pRb* can be inactivated by somatic mutations or by HPV in HNSCC<sup>139</sup>.

The facts that HPV infection is more commonly associated with oropharyngeal squamous cell carcinoma than oral cavity squamous cell carcinoma<sup>140</sup>, and that HPV-associated tumors have more favorable response to therapy<sup>141</sup> imply that these two cancer subsets are different at a molecular level. As opposed to oropharyngeal cancer, where HPV-status may be used as a prognostic marker, molecular staging to estimate the aggressiveness of OSCC is not yet viable.

As discussed above, the dynamics between cancer cells and the immune system are crucial in the outcome of OSCC. The immune balance appears to be permanently disrupted by OSCC. Higher levels of CD4<sup>+</sup> regulatory T cells have been observed in OSCC patients up to several years after successful treatment<sup>142</sup>. Alterations have also been seen in circulating NK cells of HNSCC patients<sup>143,144</sup>.

#### **2.2.4. Therapeutic interventions in OSCC**

Due to its location, OSCC patients are referred to the head and neck cancer specialist by various clinical specialists, including general practitioners, ororhinolaryngologists and dentists. Diagnosis and staging are done in concert with clinician, radiologist and

pathologist. Modern imaging modalities, i.e. computed tomography (CT) and/or magnetic resonance imaging are used routinely in disease evaluation and staging<sup>145</sup>. Furthermore, positron emission tomographic (PET) imaging is used ever more in the detection of neck metastases, as well as in post-treatment follow-up<sup>146</sup>. Lung and mediastinal metastases are also screened for by chest radiography, CT or in some cases, PET imaging. To determine the most appropriate treatment and follow-up regimen for each patient, consultation of a multidisciplinary head and neck tumor board is arranged<sup>147</sup>.

Due to the possibility of field cancerization, a pan-endoscopic examination is performed prior to surgery. Currently the gold standard in early stage OSCC is surgical treatment, combined with neck dissection<sup>148</sup>. Tumor resection needs to be sufficient to optimize loco-regional control. Current guidelines recommend that surgical margins should contain at least 5mm of histologically verified healthy tissue<sup>149</sup>. As described above, OSCC generally develops in precancerous fields. The authors of a large archive study with 827 treated OSCC patients reported that a surgical margin of up to 7mm of healthy tissue improves the survival advantage of the patients, compared to those with smaller margins<sup>125</sup>. The current recommendation is limited to a minimal margin of 5mm, which implies that a universal recommendation for healthy tissue margins that would be clearly sufficient for all patients may be difficult to define.

Adjuvant treatment is administered according to the tumor stage. Patients with locally restricted, small tumors may be treated solely by surgery, while those with more advanced tumor stages will benefit from radiotherapy<sup>150</sup>. Furthermore, concomitant chemotherapy has proven beneficial for OSCC patients with advanced stage cancer<sup>151</sup>.

One of the more recent pharmacological advances in OSCC therapy is targeting of EGFR. Excluding patients who are enrolled in clinical trials, EGFR inhibition is the only non-chemotherapeutic pharmacological cancer treatment available for head and neck cancer patients. EGFR can be inhibited by the monoclonal antibodies panitumumab and cetuximab, or by the receptor tyrosine kinase inhibitors erlotinib, gefitinib, and lapatinib. During the preparation of this thesis, four clinical head and neck cancer trials (NCT02105636, NCT01860430, NCT01935921, and NCT02110082) with different combinations of the immune checkpoint inhibiting monoclonal antibodies nivolumab (anti-PD-1), ipilimumab (anti-CTLA-4) and urelumab (anti-4-1BB), some combined with cetuximab (anti-EGFR), are active<sup>152</sup>.

### **2.3. Cancer biomarkers of the oral cavity tumors**

Biomarkers have been defined as analytical tools to assess biological parameters<sup>153</sup>. In some cases biomarkers may be prognostic, i.e. they may be utilized to estimate the risk of disease specific outcomes, such as disease progression or recurrence.

Molecular features of different tumor types are studied in the attempt to identify the primus motor for each disease. As the cellular insults leading to uncontrolled divisions may be heterogeneous, reliable means to subdivide tumors according to their



molecular characteristics are not unambiguous. Advances in biomarker studies have significantly improved the clinicians' opportunities to identify high risk patients in several cancer types<sup>154</sup>. The discovery of several unique features in various cancer types has led to novel personalized therapies, where specific cancer driving mutations are directly targeted by individually selected cancer drugs.

Canonical examples of personalized cancer therapy include certain cancer cell intrinsic mutations and growth promoting pathways. Examples include the treatment of estrogen receptor positive breast cancer with anti-estrogen drugs<sup>155</sup> or treatment of BRAF mutation-containing malignant melanoma with BRAF inhibitors<sup>156</sup>. A more recent therapeutic approach is to target PD-1-PD-L1 -mediated cancer cell immune evasion by PD-L1 blocking antibodies, as discussed above.

While PD-1-PD-L1 targeting is gaining recognition in cancer therapy, it represents only one immune evasion mechanism among many, and targeting it is not effective in all patients or all cancer types. It has been demonstrated that the recruitment of leukocytes has prognostic significance in tumors, where specific cancer associated prognostic markers are not found<sup>157</sup>. Differences in the clinical courses of patients, whose biomarker statuses are similar, may imply that their tumors are manipulating anti-tumor immune responses in yet unknown ways.

Progress in biomarker studies has been particularly rapid in breast cancer, where patients have been staged according to the expression patterns of estrogen receptor, progesterone receptor and HER2 in their tumors. The expression profiles of these immunohistochemical biomarkers have been used to select the optimal therapy for the past 10 years<sup>158</sup>. Unfortunately, comparable advances have not yet been made in OSCC, even though several potential molecules have been studied<sup>159</sup>.

This review will focus on two potential candidate biomarkers studied by us: hypoxia inducible factor (HIF)1 $\alpha$  and CD44. It will then briefly cover select candidate biomarkers, which have featured in more than one study by other groups.

### **2.3.1. Hypoxia inducible factors**

All mammalian cells need a source of oxygen to survive. For tissues, a sufficient blood supply is imperative to secure tissue oxygenation and the supply of nutrients. In order to secure cellular normoxia in conditions where cells are dividing rapidly, new blood vessels need to be formed by angiogenesis. This is evident during normal growth of young individuals or in pathological conditions such as cancer. In these cases, cells rely on efficient sensors of tissue oxygenation. The activation of angiogenetic pathways is crucial in solid tumors, but also in premalignant lesions.<sup>160</sup>

#### **2.3.1.1. Structure and function of HIF**

HIFs are master transcription factors that activate over 100 target genes in hypoxic conditions<sup>161</sup>. HIF controls and modulates oxygen delivery to the cells, and its utilization. Functionally, it acts as a transcription factor when tissue oxygenation is reduced. In some cases HIF can also activate transcription in normoxic conditions.

Generally, HIF responsive genes are activated to protect the cell from the damage caused by hypoxia.

The function of HIF1 is based on equilibrium between its production and oxygen dependent degradation. Structurally the functional form of HIF1 is a heterodimer that is composed of the stable HIF1 $\beta$ , and the labile HIF1 $\alpha$ . HIF1 $\beta$  is localized in the nucleus, whereas HIF1 $\alpha$  is localized and degraded in the cytoplasm during normoxic conditions. The degradation of HIF1 $\alpha$  is highly oxygen dependent, and is thus inhibited in hypoxic conditions. Hypoxia permits the nuclear translocation of HIF1 $\alpha$ , its dimerization with HIF1 $\beta$ , docking of this newly-formed dimer to the appropriate promoter regions and the induction of protein synthesis.<sup>162</sup>

### **2.3.1.2. Expression patterns and disease associations of HIF**

Cancer-associated hypoxia is usually the result of an insufficient blood supply, which develops when the rate of neovascularization cannot keep up with the rapid division of malignant cells. Tissue hypoxia is an important promoter of solid tumor growth<sup>163</sup>. Nuclear translocation of HIF1 $\alpha$  has been associated with angiogenesis and lymphangiogenesis in oral cavity cancer<sup>164</sup>. This has been shown to be mediated by HIF1 $\alpha$  controlled transcription of the Vascular Endothelial Growth Factor (VEGF)<sup>165,166</sup>. Lymphangiogenesis following VEGF transcription in oral cavity cancer may be at least partially caused by HIF1 $\alpha$ -mediated expression of Apelin, a small peptide with functions in several physiological processes. Apelin expression has also been shown to be prognostic for cancer recurrence<sup>167</sup>.

Tumor oxygenation status is an important predictor of the success of radiotherapy in head and neck cancer. Normoxic tumors are more radiosensitive than hypoxic tumors, which leads to fewer regional relapses during follow-up<sup>168</sup>. On the other hand, patients with hypoxic tumors have a survival advantage over those with normoxic tumors when their primary treatment has been surgery<sup>169</sup>. It should, nevertheless, be noted that these studies have been conducted on very heterogeneous patient cohorts that contain tumors of different anatomical sites and stages.

HIF1 $\alpha$  expression by cancer cells has been extensively studied in head and neck cancer (Table 1). Most of these studies have focused on the isolated expression of HIF1 $\alpha$ , and some on its combined expression along with other hypoxia related biomarkers.

**Table 1.** A summary of the previous studies on the prognostic significance of high HIF1 $\alpha$  in head and neck cancer. \*p<0.05, \*\*p<0.005, OS=overall survival, DFS=disease free survival, T1=tumor diameter <2cm, T2=tumor diameter 2-4cm.

Study	Cases	p-value	Outcome	Remarks
Uehara et al. 2009	57	*	Poorer OS	Computer-assisted scoring
		*	Lymph node metastasis	
Roh et al. 2009	43	*	Poorer OS	Only T2 staged tumors
Liang et al. 2011	89	**	Poorer DFS	
		**	Poorer OS	
		**	Lymph node metastasis	
Liu et al. 2011	112	**	Poorer DFS	
Eckert et al. 2010	80	*	Poorer OS	
Eckert et al. 2011	82	*	Poorer DFS	
dos Santos et al. 2012	66	*	Poorer DFS	
Schrijvers et al. 2008	91	*	Poorer DFS	T1 and T2 staged tumors, patients were treated only by radiotherapy
		*	Poorer OS	

### 2.3.2. CD44

CD44 is a transmembrane bound glycoprotein that plays a major role in cell migration and cell adhesion. The CD44 family consists of several isoforms, all arising as a result of alternative from the same gene. It is considered to be the main receptor for hyaluronate. Its expression is most abundant on memory-type lymphocytes, where it guides their migration, but it is expressed by most cells to some extent. In cancer, CD44 expression has been linked to disease progression in various tumor types, with partially conflicting results.<sup>170</sup>

#### 2.3.2.1. Structure and function of CD44

The CD44 gene consists of constant region exons that are used in all splice variants, and of variant exons, which may be used for the transcription and translation of several splice variants. The smallest version of CD44 (CD44s) contains only the constant region exons, while the larger family members contain different combinations of supplementary variant exons (CD44v)<sup>171</sup>. The different splice variants are differentially decorated by posttranslational modifications, such as heparan sulphate and chondroitin sulphate, but they all share the capability to bind hyaluronate<sup>172</sup>.

CD44 can also be classified as a link protein due to its ability to bind hyaluronate, similar to other important hyaluronate binding proteins such as the LYVE-1 protein expressed by lymphatic endothelial cells<sup>173</sup>. As described above, the extracellular matrix component hyaluronate plays several important roles in immunology. In addition to hyaluronate, CD44 can also bind several other extracellular or intracellular macromolecules, including other extracellular matrix components<sup>174</sup>, cell surface

receptors associated with leukocyte trafficking such as E-selectin<sup>175</sup>, heparan sulphate, matrix metalloproteinases, and cytoskeletal linker proteins.

Virtually all human cells express CD44, but at different levels<sup>176</sup>. CD44 expression may even vary in individual cells over their lifespan. Anecdotally, T cells express high levels of CD44 in their development in the thymus and after their activation by antigen exposure, but low levels during their mature but naïve state, i.e. when they are fully developed but have not yet met their antigenic epitope<sup>177</sup>.

### 2.3.2.2. Expression patterns and disease associations of CD44

CD44 has been most extensively studied in cancer<sup>178</sup>. Evidence from a mouse model of colorectal cancer implies that the expression of certain isoforms of CD44v, but not CD44s, is important in cancer initiation<sup>179</sup>. Expression patterns of CD44 have been studied in several different tumor types and with several CD44 splice variants, with conflicting results. In some studies CD44 downregulation or loss predicts cancer progression while in others, CD44 loss appears to predict improved survival. In some cancer types, but not all, CD44 expression is regulated by *p53*<sup>180</sup>.

It may thus be beneficial to carefully define the cancer type, anatomical localization and stage to maximize the impact of CD44 grading. My review of previous studies on the significance of CD44 expression by tumor cells suggests that CD44 loss may be prognostic of poorer survival only in small OSCC tumors, whereas in locally advanced tumors its prognostic significance appears to be reversed (Table 2).

**Table 2.** A summary of the previous studies on the prognostic significance of sCD44 expression in oral cavity SCC. \* $p < 0.05$ , \*\* $p < 0.005$ , n.s.=results were not statistically significant, OS=overall survival, DFS=disease free survival, T1=tumor diameter <2cm, T2=tumor diameter 2-4cm.

Study	Cases	p-value	Outcome	Remarks
Kokko et al. 2009	36 mobile tongue	n.s. trend	Worse OS in CD44 high tumors	Mixed T stages
	32 other oral cavity	n.s. trend	Worse OS in CD44 high tumors	
Huang et al. 2014	66	n.s.	No prognostic value	Mixed T stages
Oliveira et al. 2014	150	*	Better OS in CD44 high tumors	Early T stages, samples were also negative for CD133
		n.s. trend	Better DFS in CD44 high tumors	
Wang et al. 2013	66	n.s.	No prognostic value	Mixed T stages
Mostaan et al. 2011	92	**	Lymph node metastasis	Mixed T stages
González-Moles et al. 2007	46	*	Better OS in CD44 high tumors	Mixed T stages, mostly T1-2
Kosunen et al. 2007	138	**	Poorer OS with irregular CD44 staining	Mixed T stages
		**	Poorer DFS with irregular CD44 staining	
Carinci et al. 2002	20 oral cavity, 5 tonsil	*	Better OS in CD44 high tumors	Mixed T stages, mostly T1-2

Several studies have implicated CD44 to be expressed by cancer stem cells (CSC), i.e. cancer cells with a capacity to give rise to new tumors. Cell lines derived from HNSCC commonly express CD44<sup>181</sup>. CSCs resemble embryonic stem cells in many aspects, with the exception that CSCs are genetically unstable<sup>182</sup>. Currently CD44 is the most widely used marker for the identification of CSCs. Furthermore, CD44 appears to co-localize with EGFR, and it may influence EGFR-mediated signaling<sup>183</sup>.

CD44 is shed from the surface of cells to some extent *in vivo*. The levels of shed CD44 in the saliva have repeatedly been reported to be elevated in oral cavity cancer patients<sup>184,185</sup>. These studies have suggested that salivary CD44 levels could be used for the early detection of OSCC. Within cancer entities, such as HNSCC, the CD44 expression can have completely opposing impacts on disease progression, depending on the anatomical location<sup>186</sup>. Also, in some other cancer types such as lymphoma, CD44 expression by cancer cells may specifically guide lymph node metastasis<sup>187</sup>.

Soluble CD44v6 can be detected in the serum of cancer patients, but since the majority of this soluble molecule appears to be derived from leukocytes, its levels do not correlate with cancer burden<sup>188</sup>. Targeting of the splice variant CD44v6 in HNSCC by a monoclonal antibody fused to a tubulin inhibitor has been tested in a phase I trial in 30 patients. In this study conducted on incurably ill patients, three displayed a partial recovery. However, as one patient died of drug related adverse effects, the study was discontinued<sup>189</sup>. Still, the partial recovery of three incurable patients was suggestive of the potential efficiency of CD44 targeting in HNSCC, assuming that therapy-related risks could be minimized.

### **2.3.3. Other potential OSCC biomarkers**

Even though no biomarkers are currently utilized in clinical OSCC patient care, several have been studied in recent years. This review will focus on the most prominent ones. An unfortunate limitation for the interpretation of some studies is that OSCC is often grouped together with oropharyngeal tumors, despite the obvious differences between these two diseases. It is noteworthy that in most of the following studies cited, strong associations are often seen between biomarker expression and progression free or recurrence free survival, but not disease specific mortality.

#### **2.3.3.1. HPV**

The possible connection between HPV infection and oral pathology was first reported over 30 years ago<sup>190</sup>. Since then, repeated studies have shown strong correlations between infection status and response to therapy in particularly oropharyngeal tumors and pooled data from several studies demonstrates the HPV positive population to represent 20-25% of the patients<sup>191</sup>. Results from OSCC are more ambiguous since HPV is detectable in only about 2% of OSCC patients<sup>192</sup>.

#### **2.3.3.2. Tumor infiltrating leukocytes**

The host's response towards tumors has been actively studied in the recent years. The rationale behind these studies is that despite the intrinsic molecular features of

the tumor, the activity of natural defense mechanisms would be effective in limiting tumor growth.

The strongest correlations between the amounts of CD3+ or CD8+ T cells have repeatedly been associated with improved prognosis<sup>193</sup>. Intriguingly, the number of tumor infiltrating T cells predicted disease progression in HPV negative but not in HPV positive tumors in a heterogeneous HNSCC cohort<sup>194</sup>. Also, in other forms of HNSCC, the survival benefit related to HPV infection is only seen in patients with high levels of tumor infiltrating lymphocytes<sup>64</sup>. These facts may indicate that head and neck cancer aggressiveness depends, at least to some extent, on how well the immune system is able to recognize tumor cells. A small study with 39 OSCC patients replicated the results from previous uncategorized HNSCC studies. There, high CD8+ T cell counts correlated with a favorable prognosis, while high CD68+ macrophage numbers correlated with poorer clinical outcome<sup>195</sup>.

#### **2.3.3.3. p53**

Most cases of head and neck cancer are associated with mutated and defective *p53*. This is commonly caused by mutations in *p53* as such, or by HPV mediated silencing of *p53*. A study in oral cancer patients indicated that significant overexpression, which is indicative of a mutation, would correlate with poorer prognosis<sup>196</sup>. Further studies would be needed to validate this result.

#### **2.3.3.4. EGFR**

EGFR is a canonical biomarker in HNSCC<sup>197,198</sup>. It has clinical implications in various cancer types, and its neutralizing monoclonal antibody cetuximab has shown to be effective in HNSCC patient treatment<sup>199</sup>. However, therapeutic responses vary across patients and success cannot necessarily be estimated by tumoral EGFR expression<sup>200</sup>. Instead, it seems to be more important whether the downstream signaling pathways normally controlled by EGFR crosslinking are dependent on receptor ligation or if they function autonomously<sup>201</sup>.

#### **2.3.3.5. Salivary biomarkers**

Traditionally biomarkers are measured in tissue and serum samples, but recent efforts have also been put into studying the potential of sampling the saliva. This would be a particularly interesting avenue due to its non-invasive nature, but also because it could provide the clinician with near-biopsy-like information of what is taking place locally in the oral cavity, also with respect to field cancerization.

Current knowledge on salivary biomarkers is still very limited, and the most frequently studied biomarkers are inflammatory cytokines<sup>202</sup>. A proteomics approach has revealed several potential candidate biomarkers in the saliva<sup>203</sup>. Unfortunately the reference values vary greatly across the studies, and some studies have indicated serum sampling to be more sensitive than saliva sampling<sup>204</sup>.

### **3. AIMS OF THIS WORK**

Progress in cancer therapy has been tremendous during recent years, with the implementation of novel cancer biomarkers and therapies. Therapeutic success rates have increased particularly in tumors where the molecular drivers have been identified. Recent evidence derived from murine models has implied Clever-1 to be a promising cancer target in cancer, by still partially incompletely understood mechanisms. However, as cancer is such a heterogeneous group of diseases, the relevant subgroups that respond to Clever-1 inhibition should be identified. Similarly, the mechanisms involved in the therapeutic effect of Clever-1 inhibition need further exploration.

Despite the efforts of the head and neck cancer research community, assessment of OSCC aggressiveness remains difficult, partly due to the lack of OSCC specific biomarkers. Improved means to do so would be crucial in individually guiding therapeutic interventions. For these reasons, continuous optimization of diagnostics and therapy are warranted.

This work was done as a collaborative effort between the Department of Otorhinolaryngology - Head and Neck surgery of Turku University Hospital and Medicity Research Laboratory, Faculty of Medicine, University of Turku. Previous work from these two units demonstrates that Clever-1 might be a plausible therapeutic target in cancer therapy.

I focused on the following aspects:

1. How does Clever-1 deficiency influence the control of immune defense mechanisms? And furthermore, could this mechanism explain the efficiency of Clever-1 targeting in cancer? (I)
2. Could Clever-1 expression in the tumor microenvironment predict cancer specific outcome in early stage OSCC patients? (II-III)
3. Which markers could identify early stage OSCC tumors that behave more aggressively than others? (II-III)

While personalized medicine and cancer immunotherapies are finding their ways into the clinics, the answers to these questions may have implications in cancer therapy.

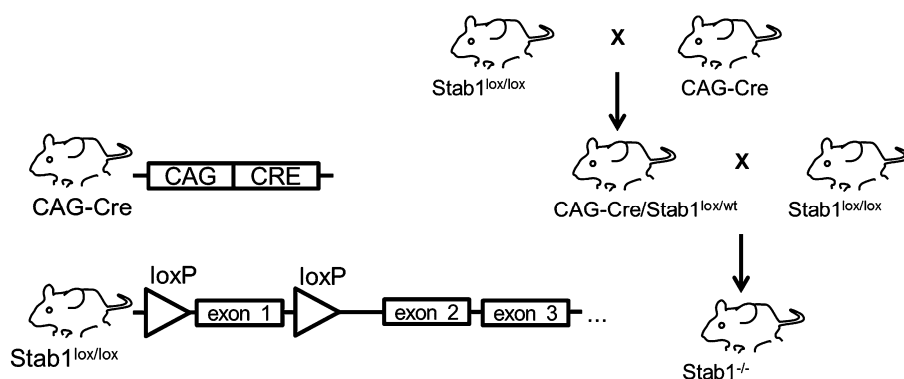
## 4. MATERIALS AND METHODS

This thesis work was conducted in both murine (I) and human (II-III) settings. All animal experiments were carried out at the central animal laboratory at the University of Turku, with respect to 3R principles: Reduction, Replacement and Refinement. Human sample collection and experiments were carried out according to Finnish legislation and the declaration of Helsinki.

### 4.1. Materials

#### 4.1.1. Murine data

Clever-1 deficient mouse strain used in (I) have been developed in house<sup>6</sup>. Briefly, loxP fragments were cloned into the (Clever-1 encoding) *Stab1* gene on both sides of the first exon. These mice were then crossed with a CAG-Cre mouse, which strongly expresses the Cre recombinase in all cells. After backcrossing, the *Stab1*<sup>lox/lox,CAG-Cre</sup> mice are devoid of Clever-1 expression (Figure 7). Wild type controls were of the *Stab1*<sup>WT/WT,CAG-Cre</sup> genotype. Clever-1 deficient and wild type control mice were age and sex matched in all experiments. All experiments were conducted at least in duplicate with a minimum of three mice per group.



**Figure 7.** Clever-1 deficient (*Stab1*<sup>-/-</sup>) mouse strain crossing scheme. Details explained in the text.

#### 4.1.2. Human data

The publications (II) and (III) use archived patient derived biopsy specimens and clinical data of oral cavity cancer patients. The biopsy specimens were collected as a part of normal patient care and clinical data. The following inclusion criteria were used: diagnoses C01-C06 in the ICD-10 classification, enough tumor-containing sample material available for histological analyses and available clinical data in hospital archives. Both studies were population based.

In (II) samples from oral cavity cancer patients treated in Turku University Hospital during 2000-2004 were used. 44 patients met the inclusion criteria and were included in the analyses of clinical disease course. Clinical data and tissue samples of



altogether 35 patients were available, and were included in the analyses concerning the correlations of biomarker profiles with clinical disease course.

Material for (III) was collected as a multi-center effort among the university hospitals of Turku, Tampere and Oulu. These samples were collected during 2000-2009, excluding the Turku University Hospital samples from 2000-2004 since these were used in the first study.

## 4.2. Methods

### 4.2.1. Immunohistochemistry (I-III)

Standard immunohistochemical protocols were used on all samples. Briefly, paraffin-embedded tissue specimens were sectioned, deparaffinized and rehydrated with descending ethanol series. The staining protocol recommended in the Vectastain reagent kit was used. DAB chromogen (Dako, Glostrup, Denmark) was used to reveal specific binding of the primary antibodies.

Multichannel immunohistochemistry was performed on OCT (Sakura, Torrance, CA) embedded frozen murine samples with Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 647 fluorochromes. The fluorochromes were either directly conjugated to primary monoclonal antibodies or the appropriate species specific secondary polyclonal antibodies. Unspecific or Fc-receptor mediated binding was blocked with serum. Antibodies are listed in Table 3.

**Table 3.** Antibodies used in the work. \*Primary antibodies were used in unconjugated, biotin conjugated or fluorochrome conjugated forms. \*\*Streptavidin binds to biotin in a non-antibody dependent manner. pAb=polyclonal antibody, IHC(P)=immunohistochemistry of paraffin sections, IHC(Fr)=immunohistochemistry of frozen sections, FCM=flow cytometry, ELISA=enzyme-linked immunosorbent assay, HRP=horseradish peroxidase

Primary antibodies used in original studies*						
Antigen	Reactivity	Isotype	Clone	Producer	Application	Used in
Clever-1	Human	Rat IgG	2-7	In-house	IHC(P)	III
Clever-1	Human, Mouse	Rat IgG2a	9-11	In-house	IHC(Fr), FCM	I
CD44	Human	Mouse IgG2a	Hermes-3	In-house	IHC(P)	II, III
HIF1 $\alpha$	Human	Mouse IgG2b	H1alpha67	ThermoFisher, Wa ltham, MA	IHC(P)	II, III
p16INK4	Human	Mouse IgG1	G175-405	Beckton Dickinson, Franklin Lakes, NJ	IHC(P)	II
Podoplanin	Human	Mouse IgG1	D2-40	ThermoFisher, Wa ltham, MA	IHC(P)	II
CD16/32	Mouse	Rat IgG2b	2.4G2	Beckton Dickinson, Franklin Lakes, NJ	FCM	I
CD19	Mouse	Rat IgG2a	1D3	Beckton Dickinson, Franklin Lakes, NJ	FCM	I
CD21/35	Mouse	Rat IgG2b	7G6	Beckton Dickinson, Franklin Lakes, NJ	FCM	I

CD23	Mouse	Rat IgG2a	B3B4	Beckton Dickinson, Franklin Lakes, NJ	FCM	I
IgM	Mouse	Rat IgG2a	II/41	eBioscience, San Diego, CA	IHC(Fr), FCM	I
IgD	Mouse	Rat IgG2a	11-26c.2a	BioLegend, San Diego, CA	FCM	I
CD169	Mouse	Rat IgG2a	3D6.112	BioLegend, San Diego, CA	IHC(Fr)	I
CD209b	Mouse	Hamster IgG1	22D1	eBioscience, San Diego, CA	IHC(Fr)	I
FITC	N/A	Goat IgG	pAb	Invitrogen, Carlsbad, CA	IHC(Fr)	I
F4/80	Mouse	Rat IgG2a	BM8	Invitrogen, Carlsbad, CA	IHC(Fr)	I
Plvap	Mouse	Rat IgG2a	Meca32	Serotec, Hercules, CA	IHC(Fr)	I

Secondary reagents and kits used in original studies						
Antigen	Conjugate	Species		Producer	Application	Used in
Rat IgG	Alexa 488	Donkey IgG	pAb	Life Technologies, Carlsbad, CA	IHC(Fr)	I
Rat IgG	Alexa 546	Goat IgG	pAb	Life Technologies, Carlsbad, CA	IHC(Fr)	I
Hamster IgG	Alexa 546	Goat IgG	pAb	Life Technologies, Carlsbad, CA	IHC(Fr)	I
Biotin**	Alexa 546	Streptavidin		Life Technologies, Carlsbad, CA	IHC(Fr)	I
Mouse IgM	HRP	Goat IgG	pAb	Southern Biotech, Birmingham, AL	ELISA	I
Mouse IgG	HRP	Goat IgG	pAb	Southern Biotech, Birmingham, AL	ELISA	I
Mouse IgG3	HRP	Goat IgG	pAb	Southern Biotech, Birmingham, AL	ELISA	I
Vectastain ABC Elite Rat IgG Kit				Vector labs, Burlingame, CA	IHC(P)	II, III
Vectastain ABC Elite Mouse IgG Kit				Vector labs, Burlingame, CA	IHC(P)	II, III
Mouse total IgG Ready-SET-Go! ELISA Kit				eBioscience, San Diego, CA	ELISA	I
Mouse total IgM Ready-SET-Go! ELISA Kit				eBioscience, San Diego, CA	ELISA	I

#### 4.2.2. Microscopy (I-III)

Patient samples in (II) were manually graded with on a conventional upright BX60 microscope (Olympus, Tokyo, Japan). Patient samples in (III) were scanned with a Panoramic 250 slide scanner and graded on a personal computer. Analyses were restricted to tumor containing areas. The CD44 grade in (III) was determined with the ImmunoMembrane plugin (Institute of Biomedical Technology, University of Tampere) for ImageJ software (NIH, Stapleton, NY)<sup>205</sup>.

Mouse samples in (I) were imaged with an LSM780 single photon confocal microscope (Carl Zeiss, Oberkochen, Germany) by sequential mosaic scanning.

#### **4.2.3. Immunizations (I)**

Mice were immunized intraperitoneally with hapten-conjugated Ficoll or keyhole limpet hemocyanin (Biosearch Technologies, Novato, CA). Blood was sampled by tail vein puncture before immunization and during the course of the humoral response.

#### **4.2.4. ELISA (I)**

Total IgG and IgM levels were measured with reagents and instructions in the commercial kits. Nitrophenyl-specific antibody reactivity in immunized serum samples was measured in an ELISA assay optimized for this work. Briefly, ELISA MaxiSorp plates were coated with NP-bovine serum albumin (BSA) and blocked with BSA. Serum dilutions were added and detected with HRP conjugated isotype specific polyclonal antibodies. TMB substrate solution (ThermoFisher, Waltham, MA) was used for color development and HCl to stop the reaction. All ELISA plates were measured on a Tecan Infinite 200 microplate reader at 450/570 nm and analyzed on Magellan software (both Tecan, Männedorf, Switzerland).

#### **4.2.5. Dendritic cell homing assays (supplementary experiments)**

Bone marrow derived dendritic cell cultures were prepared to study leukocyte trafficking to draining lymph nodes. I modified the culture protocol from previous studies<sup>51</sup>. Briefly, mouse bone marrow was collected and cultured in the presence of granulocyte-macrophage colony stimulating factor, and then stimulated with TLR agonistic lipopolysaccharides to induce maturation. Mature dendritic cells were injected s.c. and quantified in the draining lymph nodes by flow cytometry.

#### **4.2.6. FITC skin painting (supplementary experiments)**

Fluorescein isothiocyanate (FITC) mixed in carrier solution was painted on the ear skin of *Stab1*<sup>-/-</sup> or wild type mice under anesthesia<sup>206</sup>. The mice were sacrificed at pre-defined time points and FITC positive dendritic cells (which had homed to the lymph nodes from the skin) were quantified in the draining superficial cervical lymph nodes by flow cytometry.

#### **4.2.7. Flow cytometry (I)**

Single cell preparations of the studied tissues were prepared for staining by mechanical disruption, which was supplemented with enzymatic digestion when necessary. Fc-receptor mediated binding was blocked with CD16/32 specific antibodies. For intracellular stainings, cells were permeabilized with paraformaldehyde and saponin. Stainings were done on ice. The antibodies used are listed in Table 1. Data were acquired with LSRII or LSRFortessa cytometers (Beckton Dickinson, Franklin Lakes, NJ) and analyzed in FlowJo v10 software (Flowjo LLC, Ashland, OR).

#### **4.2.8. Statistical analyses (I-III)**

Data in (I) were analyzed with non-parametric tests. Single time point experiments were analyzed with the Mann-Whitney U test. In experiments with repeated measures (immunization experiments), changes from baseline values were used in the analyses.

Patient data were analyzed as follows. Relationships among immunohistochemical scores, clinicopathological variables and prognoses were evaluated. In survival statistics, disease free survival (DFS) and disease-specific survival (DSS) were analyzed over 60 months after primary treatment.

Cox proportional hazards regression model and the Kaplan-Meier method with log-rank tests were used for univariate analyses. Multivariate analyses were performed with the Cox proportional hazards regression model. Results were considered statistically significant when  $p < 0.05$ .

Statistical analyses were performed on IBM SPSS Statistics 21 (IBM, Armonk, NY) and Prism 4 (Graphpad, La Jolla, CA).

## 5. RESULTS AND DISCUSSION

This thesis has focused on two aspects: the scavenger receptor Clever-1 in the immune response and prognostic markers in head and neck cancer.

In part (I) of this work I focused on the normal function of Clever-1. More specifically, I investigated humoral immune responses in homeostatic conditions in mice that were genetically deficient of Clever-1 function. My motivation in this work came from previous work done by others, where Clever-1 was shown to limit cancer growth in murine models of melanoma, lymphoma and breast cancer by incompletely understood mechanisms<sup>6,85</sup>. The humoral immune response was important to study in Clever-1 deficient settings since its alterations might explain the anti-cancer mechanism caused by Clever-1 blockade, or conversely deficiencies therein might indicate potential therapeutic risks. In this work the loss of Clever-1 ameliorated humoral responses against the polysaccharide antigen NP-Ficoll, and to a much lesser extent towards the protein antigen NP-KLH.

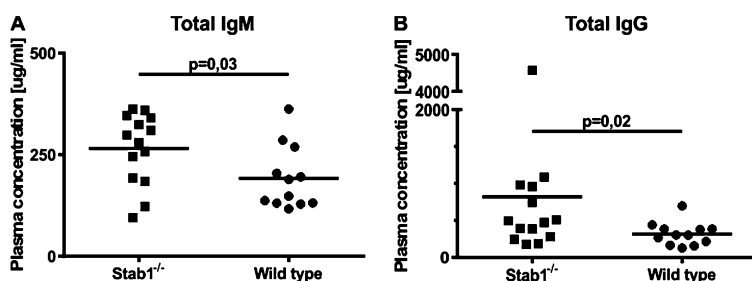
My own unpublished data had indicated that Clever-1 mediates leukocyte trafficking from the periphery to draining lymph nodes. I speculated that Clever-1 might play a similar role also in cancer, in other words it would contribute to lymph node metastasis. For this reason the prognostic effect of Clever-1 expression was evaluated in an oral cavity cancer cohort. At this point nothing was known about the role of Clever-1 in oral cavity cancer.

I conducted parts (II-III) of this work on tissue samples from oral cavity cancer patients, where prognostic values of several biomarkers on clinical disease course were examined. Contrary to my hypothesis, Clever-1 expression showed no prognostic value in these patient cohorts. However, loss of the cell adhesion receptor CD44 and the simultaneous upregulation of the hypoxia-associated transcription factor HIF1 $\alpha$  predicted cancer aggressiveness.

### 5.1. Role of Clever-1 in humoral immune responses (I)

For the potential clinical application of Clever-1 inhibiting therapies to be possible, their mechanism of action and possible adverse effects should be first understood in great detail.

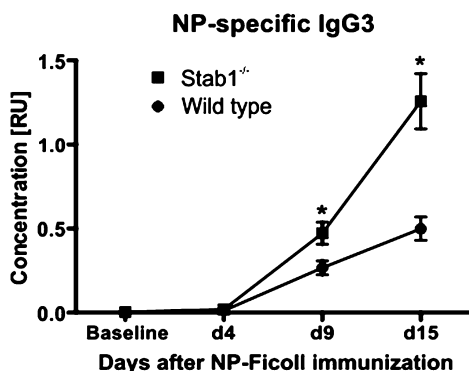
In this work I studied the role of Clever-1 in immune control, in the context of humoral immunity. The association between these two has never been studied before. This project started from the observation from a screening experiment which showed that serum antibody levels were unusually high in Clever-1 deficient (Stab1<sup>-/-</sup>) mice (Figure 8).



**Figure 8.** Spontaneously elevated antibody levels in Clever-1 deficient (*Stab1*<sup>-/-</sup>) mice. A. IgM. B. IgG. Mann-Whitney U Test.

The marked inductions in both unswitched IgM and class switched IgG antibody levels indicated accelerated humoral activity in the absence of Clever-1, which I found to be particularly interesting since such a connection has not been reported before. This observation led me to speculate that the immune system of Clever-1 deficient mice might be constitutively more active than those of their wild type counterparts. To further dissect this phenotype, I experimented on how they respond to antigen stimulation by means of immunization studies.

One of the main observations in this work was that genetic deletion of Clever-1 accelerates humoral responses particularly against polysaccharide antigens, in this case NP-Ficoll (Figure 9).



**Figure 9.** Accelerated NP-Ficoll specific IgG3 kinetics in Clever-1 deficient (*Stab1*<sup>-/-</sup>) mice after NP-Ficoll immunization. \* $p < 0.05$ , Mann-Whitney U Test.

Clever-1 deficient mice were prone to generate more robust antibody responses, which may have implied that their B cells might be positioned abnormally, or that their immune system would be under stronger immune stimulation. Clever-1 is not expressed by B cells or their progenitors, so this effect is inevitably mediated by other cell types that may influence the B cells' behavior.

The function of B cells can be influenced by Clever-1 in several ways. Their altered functions can be related to (A) abnormal trafficking behavior, (B) the availability of specific antigen stimulation, or (C) stimulation by non-antigen stimulant, such as growth factors or cytokines.

In the immunization studies, large amounts of effector cells were generated in the spleen during the course of the immune response. This made me believe the spleen might play a vital role in these events. Also the splenic MZB population was reduced in Clever-1 deficient mice, both in naïve and in antigen-stimulated conditions. It should be noted that splenectomy did not completely remove the basal differences in natural antibody levels, but it did render both genotypes unresponsive to NP-Ficoll.

Initially I speculated that splenic Clever-1 might play a role in MZB retention in the spleen, and that MZB would be mobilized from the spleen when Clever-1 function was inhibited. In this scenario, the effect of Clever-1 would be mediated by the abnormal trafficking behavior of B cells. This seemed to be the most probable hypothesis, since Clever-1 expressed by vasculature has been previously shown to play a role in leukocyte trafficking, by a potential interaction with an unknown ligand on leukocytes<sup>99,100</sup>. According to this hypothesis, Clever-1 would directly (or possibly indirectly) make contact with the B cells.

Contrary to previous experiments performed by others with anti-integrin mAb, where the inhibition of integrin function has been shown to mediate MZB trafficking<sup>42</sup>, injection of anti-Clever-1 mAb had absolutely no effect on MZB mobilization from the spleen of wild type mice in my experiments (unpublished data). These screening experiments contradicted the primary hypothesis of Clever-1 mediated B cell adhesion and trafficking, and guided the search for an alternative explanation.

I then considered if Clever-1, whose main function appears to be scavenging, might limit the access of antigens to B cell areas. I found this hypothesis to be an equally plausible explanation for the role of Clever-1 in the humoral response since structurally, Clever-1 may be classified as a C-type lectin, and may thus have the ability to directly bind the relevant carbohydrate antigens. To test this I injected fluorescently labeled carbohydrate antigen (NP-Ficoll) and tracked its distribution in vivo. Repeated studies showed no Clever-1 mediated effect on antigen distribution in the spleen, which is the major responding lymphatic organ for blood borne antigens, in either short term or long term studies. In other words, splenic B cells had similar access to antigen, irrespective of the Clever-1 genotype. Therefore, the specific mechanism by which B cell activity is affected by Clever-1 remains uncertain.

As my experiments showed Clever-1 to be a major contributor to the humoral response, but the mechanism of this effect did not seem to be explained by alterations in either leukocyte trafficking or antigen distribution, it seems likely that Clever-1 would contribute to antigen-independent stimulation of B cells.

Clever-1 has previously been studied most extensively in cancer. In mouse models of glioblastoma multiforme and more recently in those of breast cancer, Clever-1 is most abundantly expressed by macrophages in early cancer stages, and it is subsequently downregulated as the tumors progress<sup>85,103</sup>. Here I demonstrated that genetic knockout of Clever-1 leads to accelerated humoral responses in mice. Although the

molecular mechanisms leading to overactive humoral responses in Clever-1 knockout mice remain to be elucidated, I will speculatively discuss a few possible scenarios.

## **5.2. Mechanistic options for Clever-1 function in cancer**

Evidence supporting the importance of Clever-1 in cancer is accumulating. Clever-1 expression is upregulated in tumor associated macrophages and vasculature, including head and neck cancer, and vessel positivity correlates with lymph node metastasis<sup>7</sup>. Clever-1 inhibition has been associated with fewer regulatory T cells in murine tumor models<sup>6</sup>.

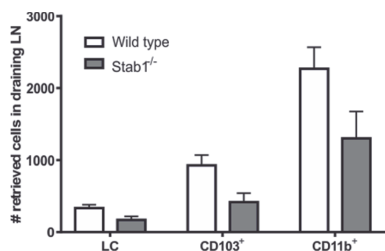
Taking into consideration the conflicting prognostic value of SPARC, a Clever-1 ligand, in previous studies<sup>207,208</sup>, it seems unlikely that Clever-1 expression would have an unambiguous prognostic role in all tumors. This matter should be seriously considered when results from studies on the prognostic role of Clever-1 are evaluated. For instance, our results suggest that Clever-1 might not be a promising prognostic biomarker in early stage OSCC (II-III).

The primary proposed mechanism of action of Clever-1 inhibiting therapies is related to leukocyte trafficking. Previous work has suggested that Clever-1 plays a role in leukocyte transmigration through blood and lymphatic vasculature<sup>97</sup>. In brief, the recruitment of regulatory T cells into tumors is to some extent mediated by Clever-1, and the inhibition of Clever-1 function by monoclonal antibodies or genetic manipulation compromises their accumulation in tumors<sup>6</sup>. In addition to this primary proposed mechanism of selective T cell recruitment to tumors, supplementing mechanisms may coexist.

Since Clever-1 is expressed by tumor associated lymphatics, it seems plausible that it might also affect cancer cell trafficking towards draining lymph nodes, and thus play an active role during metastasis. It should be noted that cancer cell trafficking is quite difficult to study *in vivo*, for the obvious reason that cancer cells move fairly slowly. Leukocytes migrate through the tissue at markedly faster velocities, with partially overlapping mechanisms, and they are used in studies to make some extrapolations also applicable for cancer cells. For this reason, I used bone marrow derived dendritic cells to study the effect of peripheral Clever-1 expression on trafficking of leukocytes towards draining lymph nodes.

Dendritic cell homing to draining lymph nodes was markedly impaired in Clever-1 deficient mice in dendritic cell injection experiments (unpublished data). Supplementing experiments showed that this phenotype was clear with all major cutaneous dendritic cell subsets, namely with Langerhans cells, CD103<sup>+</sup> dermal dendritic cells and CD11b<sup>+</sup> dermal dendritic cells (Figure 10, unpublished data). Since dendritic cells and cancer cells both utilize the lymphatic vasculature to reach the lymph nodes, I speculated this phenotype might also be applicable to cancer cell metastasis. This notion was supported by the knowledge that Clever-1 expression is induced in the tumor stroma, which might be a way to promote metastasis.





**Figure 10.** Attenuated dendritic cell trafficking in Stab1<sup>-/-</sup> mice in the FITC skin painting experiment. LC = Langerhans cells, CD103<sup>+</sup> and CD11b<sup>+</sup> = the major dermal dendritic cell subsets.

Functionally Clever-1 is a highly multifunctional molecule, so supplementing pro-tumor effects should be also considered. Immunosuppressive functions have been shown to be mediated by Clever-1 and its inhibition may skew the immunological microenvironment towards a pro-inflammatory direction<sup>96</sup>. The inflammatory milieu associated with cancer has been acknowledged since the 1950's, and pro-inflammatory therapies, such as intravesical application of the tuberculosis vaccine BCG, have routinely been used in the clinics for decades.

Mechanistically cancer rejection mediated by Clever-1 inhibition may be dependent on T cell activity. It has been well established that tumor rejection is highly dependent on IFN- $\gamma$  and its receptor<sup>209</sup>, and recently Palani and colleagues have shown Clever-1 inhibition to ameliorate the T cell's ability to secrete interferon gamma in response to antigen in *in vitro* co-culture experiments<sup>96</sup>. Another previous report has described that Clever-1 may be a particularly important leukocyte trafficking receptor for regulatory T cells in the liver<sup>99</sup>. I consider these functions to be possible also in cancer, and in my opinion further work looking into them would be warranted.

Alternatively, the notion that the anti-tumor effect of Clever-1 inhibition would be partially attributed to B cells may be an equally plausible explanation. Tumor specific antibodies in cancer patients have been identified 40 years ago<sup>210</sup>, and host-derived anti-tumor antibodies have been shown to mediate direct cancer cell killing<sup>211</sup>. Their mechanism of action may be related to antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP). In ADCC, NK cells and other cytotoxic cells are activated after Fc receptor ligation<sup>212</sup>. It has been reported in cancer patients treated with tumor-specific mAb in clinical trials, that in addition to their role in blocking receptor ligation of their target cells, the antibody administration clearly correlates with leukocyte recruitment to tumors<sup>213</sup>. In ADCP, Fc receptor ligation on phagocytes, such as tumor associated macrophages, triggers their phagocytic activity. Finally, surface-bound antibodies may activate the complement system.

My work showed that antibody production is accelerated in genetically Clever-1 deficient mice. While this matter has not been studied in tumor bearing mice, it would certainly be an interesting path to follow. Whether or not this phenomenon can be seen after pharmacological inhibition of Clever-1 function remains to be elucidated. Also, diversity of the antibody repertoire (capacity for somatic antigen receptor editing) in Clever-1 deficient mice is to-date unknown.

It should also be kept in mind that the Clever-1 ligand SPARC has also been shown to be associated with cancer growth in various different tumors<sup>214</sup>, and that SPARC inhibition has a therapeutic effect at least in some cancer types<sup>215</sup>. A recent report has demonstrated the importance of Clever-1 mediated scavenging of SPARC in the context of breast cancer<sup>85</sup>.

In conclusion, the anti-tumor effect exerted by Clever-1 inhibition most likely has multiple contributing factors, and it might be related to altered functions of B cells, T cells and stromal cells alike.

### **5.3. Prognostic markers in stage 1 oral cancer (II-III)**

Functional properties of cancer cells can be crudely divided according to the classical cancer hallmarks (Figure 3). For research and diagnostic purposes, these hallmarks can be studied and quantified with the help of biomarkers, which are typically proteins, or noncoding RNAs, that may be overexpressed, downregulated or mutated.

It is left to the discretion of the scientific community to evaluate how well a given biomarker represents any given cancer hallmark. Also, due to the biological functions carried out by the studied hallmark-associated biomarkers, many of them may factually represent more than one hallmark.

The molecular pathology of OSCC is currently not routinely evaluated in the clinics for prognostic purposes. Therapeutic decision making is based on staging made according to clinical, pathological and radiographic parameters. Information on the tumor microenvironment might assist the clinicians in delivering optimal therapy for each patient, and for this reason suitable histopathological biomarkers would be needed.

I started my pilot study by reviewing the literature for suitable candidate biomarkers. My objective was to screen for immunohistochemical biomarkers from more than one cancer hallmark. In the same context I wanted to weigh the usefulness of Clever-1 as a prognostic biomarker in OSCC, which has never been done before. The ultimate aim in this study was to identify a suitable panel of biomarkers, which could potentially be used to stratify patients according to their risk of cancer recurrence.

The pilot material in my studies consisted of paraffin-embedded tissue material from OSCC patients diagnosed with stage 1 disease, who were treated at Turku University Hospital during the years 2000-2004. During this time interval, according to contemporary views on OSCC care, the patients' treatment protocols were limited to tumor excision. Since it was soon noted that less aggressive therapy yielded unacceptably poor therapeutic success rates, clinical protocols have been extended.

Currently, treatment guidelines of T1-2N0M0 OSCC feature cervical sentinel lymph node biopsy or complete removal by elective neck dissection, at the very least<sup>149</sup>. While trends in clinical practices for minimal treatment of stage 1 OSCC patients were

soon taken back as unethical, this pilot provided clinical samples where the effect of therapy was minimal.

Based on an extensive review of the literature, I focused on the following biomarkers: Ki67 as an indicator for proliferative capacity, CD44 and podoplanin as indicators of epithelial-mesenchymal transition, HIF1 $\alpha$  as an indicator of metabolic switch, p16<sup>INK4a</sup> as an indicator of a transcriptionally active HPV infection, and Clever-1 as a tumor microenvironment associated scavenger receptor and potential adhesin, with a potentially broad spectrum of immunological functions. The background and results of my studies are discussed below.

### **5.3.1. Ki67**

Previously it has been shown that highly proliferative (Ki67 positive) oral cavity squamous cell tumors, that simultaneously contain p53 overexpression, are highly aggressive<sup>216</sup>. High Ki67 labeling indices have also been observed in primary OSCC that had already metastasized<sup>217</sup>.

Such clear correlations were not seen between the proliferative capacity of cancer cells, measured here by Ki67 labeling index, and clinical parameters, in the primary cohort (II). Even though the pilot cohort was reasonably small, statistically non-significant results on the predictive value of Ki67 staining imply that other markers may be better predictors of disease aggressiveness.

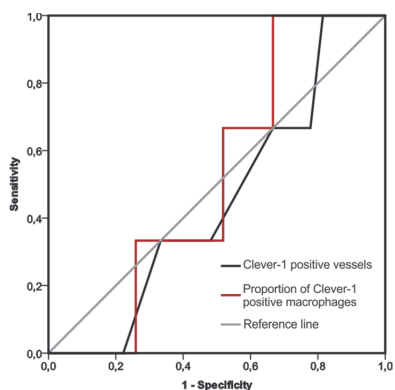
Previously high cancer cell proliferation rate has been shown to be associated with poorly differentiated OSCC tumors, but not tumor size. In this study, any independent prognostic value of Ki67 immunohistochemistry was not seen<sup>218</sup>. Two large meta-analyses on the predictive value of proliferative markers have been published. The former contained studies published before 2003<sup>219</sup>, and the latter contained studies from 2005-2009<sup>220</sup>. Both of these meta-analyses contained studies with conflicting results, which, along with my results, implies that Ki67 staining results may not be repeatable enough for clinical applications. For these reasons Ki67 analyses were omitted from my second cohort.

### **5.3.2. Clever-1**

Clever-1 appears to play a role in promotion of tumor growth in murine models of melanoma, T cell lymphoma and breast cancer<sup>6,85</sup>. Additionally, my own results showed that Clever-1 mediates leukocyte trafficking from the periphery towards draining lymph nodes, a feature which might be shared by cancer cells. If so, Clever-1 might be a relevant prognostic factor in lymph node metastasis. For these reasons I studied the role of Clever-1 in two OSCC patient cohorts (II-III, results from II unpublished). In these analyses, I used a new highly specific rat anti-human monoclonal antibody (2-7 clone), which has been developed in-house, to identify Clever-1 expression in these samples.

The prognostic role of Clever-1 was first studied in a relatively small cohort that was collected for preliminary screening of suitable biomarkers. From this staining, Clever-1

positive macrophages were quantified as their proportion of all CD68<sup>+</sup> macrophages. Clever-1 positive vessel density was quantified in total. Both parameters were then analyzed with five year disease free survival as the readout (Figure 11).



**Figure 11.** Receiver operating characteristic curve assessing the prognostic significance of Clever-1 on OSCC recurrence. Black line = impact of Clever-1 positive vessel density, red line = impact of Clever-1 positive macrophage proportion, grey line = reference. Results n.s.

The results from this small pilot study with samples from 35 patients indicated that Clever-1 expression might not to be clinically prognostic in stage 1 OSCC. In this study Clever-1 positive vessels and macrophages were separately analyzed, but no association between staining patterns and clinical outcome was observed.

Clever-1 expression by tumor associated macrophages has previously been shown to be linked to less aggressive tumors in colorectal cancer patients<sup>87</sup>, but no association is seen in bladder cancer<sup>104</sup>. Due to this discrepancy in previous results and the relatively small amount of samples available in my pilot study, I was not yet completely convinced that Clever-1 expression would have no impact on OSCC prognosis. For this reason I wanted to apply the same analyses to the larger OSCC validation series I had at hand for other analyses.

I analyzed the tumor associated expression of Clever-1 in the larger validation series, which consisted of 171 OSCC patients and then correlated these results to the clinical outcomes of these patients. Careful analysis of this validation dataset repeated my previous result that Clever-1 is not a useful prognostic indicator in stage 1 OSCC (III). I conclude that at least in early stages of OSCC, Clever-1 is not a promising prognosticator. Nevertheless, tumor growth is attenuated by both pharmacological and genetic Clever-1 inhibition in mice<sup>6,85</sup>, which implies that much still remains to be learned about the biological function of Clever-1 in cancer. To my knowledge, Clever-1 targeting in oral cancer models has not been studied.

The work (II-III) presented in this thesis was focused on early stage oral cavity squamous cell carcinoma, which is biologically distinct from the other cancer types where Clever-1 may have better prognostic potential. For instance, the report by Ålgars and colleagues showed Clever-1 expression to have the prognostic potential only in late, but not early stages of adenocarcinoma in the colon<sup>87</sup>. Late stages of OSCC were not included in my material, so the possible prognostic role of Clever-1 in advanced OSCC cannot yet be addressed.

Even though Clever-1 is strongly upregulated in the tumor microenvironment in some tumors<sup>7</sup>, it is most abundantly expressed by sinusoidal endothelial cells of the spleen, liver and bone marrow<sup>82</sup>. Also, work with murine implanted tumors has implied Clever-1 to be only transiently expressed during a specific early phase in tumor progression<sup>85,103</sup>. Possibly, the role of Clever-1 in cancer is not limited to its functions in the tumor microenvironment, but it may also exert its effects systemically. For this reason it would be interesting to see if pharmacological or genetic Clever-1 inhibition should have any therapeutic effect in oral cancer. A potentially suitable model to study this question would be the 4NQO model, where oral squamous cell carcinoma is chemically induced in rodents<sup>221</sup>.

The role of Clever-1 in cancer progression may be related to the clearance of SPARC, a tumor associated protein, for which Clever-1 is the only known receptor. SPARC is strongly expressed in tumors and it has been characterized to be associated with gastric cancer progression in patients<sup>222</sup> and with cancer cell invasiveness in vitro<sup>223</sup>. Conversely, another study conducted with ovarian cancer patients directly opposed these results, and suggested that higher SPARC expression was associated with more favorable clinical outcome, and backed these data up with in vitro results that showed that cancer cell line invasiveness could be inhibited by SPARC. Data from the same study showed that SPARC expression clearly decreases in more advanced T stages<sup>224</sup>.

Evidently the aforementioned studies suggest that either the cancer-associated function of SPARC is highly cell type specific, or that results from either study should be reinterpreted. Studies from a genetically SPARC deficient mouse strain suggest that SPARC may in fact have completely opposing roles either as a tumor suppressor in some tumors, such as murine pancreatic cancer<sup>225</sup> and prostate cancer<sup>207</sup> or as a tumor promoter in peritoneally disseminated ovarian cancer<sup>226</sup> or colorectal cancer<sup>208</sup>. The inhibition of Clever-1 genetically or pharmacologically could hypothetically result in SPARC accumulation in the tumor microenvironment, but this should be further studied in several different cancer types.

As a functional alternative, the genetic deletion of SPARC has been reported to accelerate leukocyte trafficking from the periphery to draining lymph nodes<sup>227</sup>. In this report the authors hypothesized, but did not directly show, that dendritic cell interstitial migration in the skin is attenuated by SPARC due to its role in forming or maintaining the dense architecture of the interstitium. These events may also be applicable to cancer cells, which use the same routes in metastasis, as leukocytes use in their physiological trafficking behavior. If this is the case, Clever-1 may contribute to this phenomenon since it is the main receptor for SPARC.

Arguably, the role of Clever-1-SPARC interaction is likely to contribute to tumor progression or regression. The importance of this interaction has been suggested in breast cancer in a recent publication that contains data from both patient material as well as a murine disease model. Interestingly, also these results showed that Clever-1

(referred to as stabilin 1 in the publication) is most abundantly expressed in the earliest and latest stages of cancer, but down regulated in intermediate stages<sup>85</sup>.

### 5.3.3. CD44

The predictive value of CD44 has previously been observed by others, and as described above, several CD44 splice variants exist. Partially conflicting prognostic values for the CD44 standard form, as well as for CD44 splice variants, have been reported (reviewed in by Naor et al.<sup>173</sup>).

CD44 expression had a predictive trend with the disease course in my pilot study (II). The patients with low CD44 expressing tumors were at a considerable risk of cancer recurrence. Disease-specific mortality (cancer associated death during the follow-up) was not statistically predicted by CD44 score. Since the expression of CD44 has previously been noted as an unfavorable prognostic indicator, I suggest interpretation of CD44 staining results in future studies should be done with care. It appears that the predictive value of CD44 expression is reversed during the course of the disease (see Table 2). Similarly, CD44 expression appears to have a site-specific prognostic role in HNSCC<sup>186</sup>, which implies that HNSCC of different sites may have very different molecular features.

CD44 is a molecule with various functions, only one of which is adhesion. Its functional role in the beginning of oncogenesis may be very different from those in more advanced stages. Speculatively the adhesive role of CD44 may be crucial in early stage tumors, and its loss would result in cancer cell detachment and earlier metastasis. In later stages of cancer progression, the role of CD44 on e.g. EGFR signaling may overdrive its importance on the cancer cell's adhesive properties, and thus it would start to promote cancer progression in this model. This potential CD44-EGFR mediated signaling (or any other equally important affected pathway) could be important for the self-sustaining replicative cycle associated with cancer stem cells, and it might lead to cancer aggressiveness.

### 5.3.4. Podoplanin

Podoplanin is a well-established marker for lymphatic vessels, which are also the primary route for OSCC metastasis<sup>228</sup>. The results from my pilot study showed no major prognostic value in podoplanin immunohistochemistry in terms of podoplanin positive lymphatic vessel density or cancer cell associated podoplanin expression (II). These results have been replicated in an independent study with a larger cohort of OSCC patients<sup>229</sup>. With repeated negative results, it is unlikely that podoplanin would prove to be a useful prognostic biomarker in future studies and for these reasons I omitted the staining from my validation study.

### 5.3.5. p16<sup>INK4a</sup>

I used p16<sup>INK4a</sup> immunohistochemistry to screen for HPV-association in the pilot study. The specificity of this method has been a subject to some critique among the HPV-researchers' community, but it is still considered an acceptable and relatively cost-effective method for rather general analyses of HPV infection in head and neck

squamous cell carcinoma since it may be used to identify all HPV-subsets<sup>230</sup>. However, a more recent prospective study conducted with OSCC patients specifically, showed poor correlation between p16<sup>INK4a</sup> expression and HPV16/18 PCR, and no significant value for p16<sup>INK4a</sup> as a prognosticator<sup>231</sup>.

Nevertheless, only two samples in my pilot study were p16<sup>INK4a</sup> positive. With such a low incidence rate, any conclusions cannot be made in terms of prognosis. For this reason, I omitted p16<sup>INK4a</sup> immunohistochemistry from analyses of the second cohort.

### **5.3.6. HIF1 $\alpha$**

Results from my pilot study (II) showed that strong HIF1 $\alpha$  expression in stage 1 OSCC was associated with an elevated risk for cancer recurrence. Similarly, the cancer-specific survival, which accounts for only cancer-related deaths, tended to be poorer in patients with high HIF1 $\alpha$  expressing tumors.

According to the results, primarily resected tumors with high HIF1 $\alpha$  expression would behave more aggressively. These results are in apparent conflict with a previous report by Beasley and coworkers<sup>169</sup>, but may be explained by the fact that my patient cohort was much more homogenous in terms of tumor site (all from the oral cavity) and T stage (all T1), whereas the report by Beasley and coworkers did not contain subset analyses by tumor site or T stage. A supplementary subset analysis of their material would be particularly intriguing to see, since their completely opposing results might indicate that in another tumor site or T stage, HIF1 $\alpha$  upregulation would play an even stronger, but opposite role.

### **5.3.7. Combination of CD44 and HIF1 $\alpha$**

Since two of the studied biomarkers, namely CD44 and HIF1 $\alpha$ , turned out to be particularly promising, I decided to analyze their combined effect. These two biomarkers represent two different cancer cell hallmarks, namely the cancer cells' adhesive properties and metabolic switch, respectively. Their effects may partially overlap in cancer, since CD44 has previously been reported to be regulated by HIF1 $\alpha$ <sup>232</sup>.

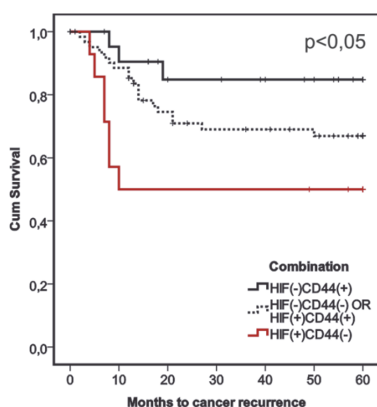
A strong predictive tool was evident when CD44 and HIF1 $\alpha$  stainings were combined (II). Poor outcome was observed with CD44 negative, HIF1 $\alpha$  positive tumors. Inversely those with CD44 positive, HIF1 $\alpha$  negative tumors remained in remission five years after treatment. A similar trend, which did not reach statistical significance, was apparent in disease-specific survival.

Encouraged by the preliminary results, a larger collaborative study using the same markers was performed (III). This was necessary in order to validate results from the pilot study. To this end, two other head and neck cancer centers, namely the head and neck surgery departments at the university hospitals of Tampere and Oulu were recruited in the study, and a cohort of 171 patients was collected. As the pilot study (II) indicated CD44 and HIF1 $\alpha$  to be the most promising prognosticators of cancer

aggressiveness of the biomarkers, the other markers except for Clever-1 were omitted from this validation series (III).

Analyses in the validation study were partially supported by computer assisted scoring, where the CD44 staining intensity was quantified semi-automatically. This refinement of the analysis made the results more reliable as they were more objective.

The results from this validation series indicated that clinical disease course is more accurately combined when several biomarkers are simultaneously studied (III). The worst outcome was seen in patients with CD44 negative, HIF1 $\alpha$  positive tumors. While the results were slightly less evident in this validation cohort than they were in the pilot cohort, a very clear difference between the most aggressive and least aggressive tumors was observed (Figure 12).



**Figure 12.** Prognostic significance of combined HIF1 $\alpha$  and CD44 expression, validation series (III). HIF1 $\alpha$ -CD44<sup>+</sup> tumors are depicted with a black solid line, HIF1 $\alpha$ -CD44<sup>-</sup> and HIF1 $\alpha$ <sup>+</sup>CD44<sup>+</sup> are depicted with a black dotted line, and HIF1 $\alpha$ <sup>+</sup>CD44<sup>-</sup> tumors are depicted with a red solid line.  $p < 0.05$ , log-rank test.

The work presented in this thesis and the enclosed original publications suggests that OSCC aggressiveness might be predictable with the help of biomarkers, but these results still demand validation by a prospective study before their clinical application may be considered. This study was focused on CD44 and HIF1 $\alpha$ , two markers I considered promising and appropriate. For technical reasons concerning statistics, the sample sizes allowed analyses of only one or two markers simultaneously. Larger studies with more samples and more markers may yield even better predictive potential, with even more accurate predictive potential.

Even though CD44 and HIF1 $\alpha$  seemingly represent rather straightforward properties in tumors, namely their adhesive properties and responses to hypoxia, they both are in fact quite multifaceted molecules (discussed above in more depth). For these reasons, significant changes in their expression levels may be indicative of a multitude of pathologically significant events. Analyzing the expression levels of both markers will presumably give us a fairly good idea on the prognostically relevant molecular events taking place in the tumor and its microenvironment. It seems plausible that alterations in two separate molecular pathways would indicate more profound molecular pathology in the tumor. Interestingly, using the combination of more than one variable to prognosticate oral cavity cancer has shown great promise also in work published by



others. As an example, Almagush and colleagues combined the depth of invasion with the invasion pattern to identify those patients at high risk<sup>132</sup>.

### **5.3.8. General considerations in cancer immunophenotyping and risk assessment**

Analysis of a multitude of features in tumors is feasible with modern technologies, including next generation sequencing. Theoretically, an almost infinite amount of data can currently be obtained from tumors for a reasonable amount of money. Our main limitation is data processing and implementation to patient care. For practical clinical decision making, the interpretation of molecular diagnostic and prognostic biomarkers should be as straightforward and dichotomic as possible.

Combining variables (e.g. biomarkers) for analyses always increases the number of groups exponentially. In other words, by analyzing one biomarker at a time patients are divided into two groups, two biomarkers divide patients into four groups and three biomarkers will divide patients into eight groups, in an exponential manner. According to this principle, the simultaneous analysis of three biomarkers would inevitably require hundreds of patients, and four simultaneous biomarkers might require more than a thousand patients for these studies to be feasible and fruitful<sup>233</sup>. For this reason, it is very seldom that more than two markers can be studied at the same time.

Before novel biomarkers can be implemented in clinical decision making, results need to be cross validated on several levels. These biomarker studies consisted of independent datasets, which is one of the gold standards of quality. If the results from these independent studies are similar, as they were in (II) and (III) of this thesis, a further prospective study should be conducted. A prospective study to validate the results is being planned as this thesis is being written.

Several issues need to be considered when multi-parameter risk assessment panels are implemented in clinical settings. Increasing data points may be difficult for clinicians to incorporate in currently practiced decision making schemes. Novel biomarkers may also strain the efforts of diagnostic units, usually the departments of pathology and radiology, since the interpretation of an increasing load of biomarkers and studies demands continuous training and increases the work load. Fortunately, technological innovations may be used to overcome these challenges. Semi-automated diagnostic tools may supplement the expertise of diagnosticians. Also, digital image files provide improved possibilities for inter-hospital, even international consultation. Finally, the interpretation of these increasing datasets following each patient may be assisted by multi-parameter risk calculators and similar tools, to provide more accurate risk assessment possibilities.

Despite the continuous extensive study of various biomarkers in various studies, the clinical implementation of these biomarkers has been sluggish. Cancer biomarker studies have historically yielded heterogeneous results across studies. A major concern, which urgently needs to be addressed, is the fact that standardized protocols in immunohistochemical scoring are generally lacking. In practice this means that

results between two similar studies, conducted on similar patient cohorts with the same biomarkers are not necessarily comparable, and they may in extreme cases give completely opposite results due to different analytical protocols.

An absolute prerequisite for the implementation of novel biomarkers is a consensus on the classification of staining results. Previous universal classification guidelines for biomarkers that have made their way to the clinics have been achievable only when sufficient number of studies had been conducted with the same classification criteria. These prior studies have then been subjected to a meta-analysis, where the results have been processed and a new consensual result is formed.

In the best case scenario, the expert committee defining classification guidelines should have access to the complete raw data, i.e. the stained histological specimens and patient data. These datasets would enable the committee to make their own, unbiased next generation meta-analysis of the previous studies, and would increase the probability of reaching a decision. With the complete raw data, this committee could possibly even re-evaluate the data from studies, where false conclusions may have been made due to small sample sizes, unfortunately chosen diagnostic cutoff values, or inappropriate statistical methods.

With recent technological and organizational development, such as high throughput microscopes, sophisticated computerized algorithms, biobanks containing escalating amounts of tissue material, tumor microarrays and clinical data, along with the increasing demands for transparency in scientific reporting, the prerequisites for these next generation meta-analyses exist. If immunohistochemical protocols in different studies have been similar, these new advances may open completely new horizons in biomarker implementation. In addition to the improved possibilities to re-analyze results from previous studies, these advances may also accelerate the output in the future.

Current biomarker studies are conducted with a high emphasis to manual data acquisition. This data acquisition workflow consists of collecting tissue material from archives, their processing in the staining protocol, inspection under a light microscope, and manual scoring. The completion of this workflow may easily take weeks, if not months to complete, and it obviously hinders all other forms of productivity of the researcher, who is usually also a clinician.

One of the most dramatic recent advancements in artificial intelligence is the ability of computer programs to recognize patterns. This has successfully been tested in the semi-automated diagnosis of malaria from digitalized blood smear samples, where an algorithm screened thousands of fields of view and cherry picked the ones where potentially infected red blood cells were detected<sup>234</sup>. While cancerous tissue may be more difficult for computer programs to identify, it will most likely be possible in the very near future. In an interesting related recent study, domesticated and trained pigeons were reported to be able to reliably recognize breast cancer, but not

dysplasia, in digitalized histological specimens and mammograms. This study implied that the recognition of cancer by pattern is a feature that can be easily trained.<sup>235</sup>

A possible future tool used for diagnostic and prognostic assessment of cancer patients might be an algorithm where all of the known parameters concerning the patient would be entered. These parameters would contain patient-related information, such as gender, age and prior illnesses, clinical information concerning the tumor, such as TNM stage and anatomical site, and cancer phenotypic data, such as results from biomarker or radiological analyses. This hypothetical algorithm would then assist the clinician in choosing the optimal therapeutic approach to the patient. Such algorithms are already used in cardiovascular disease risk assessment.

## 6. CONCLUSIONS AND FUTURE PERSPECTIVES

### 6.1. Role of Clever-1 in immune balance

The majority of previous studies concerning Clever-1 have focused on its scavenger function on macrophages, while only few papers to date have dissected its role on the vasculature<sup>97,100</sup>. The work in this thesis showed that humoral immune responses are affected by the genetic ablation of Clever-1. In unstimulated settings, Clever-1 expression is limited to sinusoidal endothelial cells e.g. in the splenic red pulp, which implies that these endothelial cells may be important in the control of the humoral response.

The most likely means by which Clever-1 might control B cell responses may be related to increased unspecific stimulation of B cells, possibly by cytokines or by waste molecules that Clever-1 would normally scavenge. This is obviously speculative and also difficult to show experimentally since the cytokine signaling network is very complex. Even though the precise mechanism by which Clever-1 controls immune responses remains unknown, no major concerns regarding the safety of its therapeutic blocking in cancer exist, at least according to our current knowledge.

### 6.2. Combined CD44 and HIF1 $\alpha$ expression levels predict clinical outcome in OSCC

The results presented in this thesis proposed that combining the results from CD44 and HIF1 $\alpha$  immunohistochemistry might be a promising way to evaluate the prognosis of early stage OSCC. Speculatively these two biomarkers may be particularly good prognosticators since they are both involved in several tumor-associated processes, and their expression levels may presumably be altered by a broad range of tumor promoting events. A prospective validation study is currently being planned. In case these prospective analyses give similar results, CD44 and HIF1 $\alpha$  guided risk assessment should be considered in pathology departments and the clinics.

### 6.3. Prognostic role of Clever-1 in OSCC

Along with CD44 and HIF1 $\alpha$ , I studied the prognostic and functional role of Clever-1 as it is still a novel and incompletely understood scavenger receptor associated with cancer. The biomarker studies I presented in this thesis showed no predictive potential for Clever-1 expression on either lymphatic vessels or macrophages. This, and the arguably thin evidence for its prognostic value in colorectal cancer<sup>87</sup> and bladder cancer<sup>104</sup>, imply that other biomarkers may be more valuable to guide clinical decision making at least in some tumors. Even though the prognostic significance of Clever-1 expression may be ambiguous, the data to support its therapeutic potential is more straightforward.

It should be noted that since biological processes such as protein synthesis always require large amounts of energy, it is very seldom they take place in vein. This in mind, the induced expression of Clever-1 in tumor associated vasculature is likely to have significance in biological processes and attempts to elucidate them should be made.

It may either be an attempt by the host to fight against the tumor, or it may be induced by the tumor for e.g. immune evasion. Future work to explore the pro-tumor effect mechanistically in more detail would be warranted since such information could clarify which pathological processes we might be able to treat by targeting Clever-1.

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