Subgroups of Merkel Cell Carcinoma Patients Defined by Young Age, Immunodeficiency and Polyomavirus Infection

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ACADEMIC DISSERTATION

To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki, in the Biomedicum Lecture Hall III (Haartmaninkatu 8) on November 7th 2014 at one o’clock in the afternoon.
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<tr>
<td>aCGH</td>
<td>array comparative genomic hybridisation</td>
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<tr>
<td>AD</td>
<td>autoimmune disease</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ATC</td>
<td>anatomic therapeutical classification</td>
</tr>
<tr>
<td>BCC</td>
<td>basal cell carcinoma</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-Cell CLL/Lymphoma 2</td>
</tr>
<tr>
<td>CK-20</td>
<td>cytokeratine 20</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>DMI</td>
<td>diabetes mellitus, type I</td>
</tr>
<tr>
<td>DMII</td>
<td>diabetes mellitus, type II</td>
</tr>
<tr>
<td>FCR</td>
<td>the Finnish Cancer Registry</td>
</tr>
<tr>
<td>GIST</td>
<td>gastrointestinal stromal tumour</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>ICD</td>
<td>International Statistical Classification of Diseases and Related Health Problems</td>
</tr>
<tr>
<td>LT</td>
<td>large T-antigen of Merkel cell polyomavirus</td>
</tr>
<tr>
<td>MC</td>
<td>Merkel cell</td>
</tr>
<tr>
<td>MCC</td>
<td>Merkel cell carcinoma</td>
</tr>
<tr>
<td>MCV</td>
<td>Merkel cell polyomavirus</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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</table>
MS-MLPA  methylation-specific multiplex ligation-dependent probe amplification
NMSC  non-melanoma skin cancer
NK cells  natural killer cells
OR  odds ratio
PCR  polymerase chain reaction
qPCR  quantitative polymerase chain reaction
RA  rheumatoid arthritis
RB  retinoblastoma protein
SCC  squamous cell carcinoma
SEER  the Surveillance, Epidemiology and End Results program
SII  the Social Insurance Institution of Finland
SIR  standardized incidence ratio
SLNB  sentinel lymph node biopsy
ST  the small T-antigen of Merkel cell polyomavirus
TIL  tumour infiltrating leukocyte
Treg  regulatory T-cell
TTF-1  thyroid transcriptor factor 1
UVR  ultraviolet radiation
VLP  virus-like particle
95%CI  95% confidence interval
ABSTRACT

Background and purpose: Merkel cell carcinoma (MCC) is a rare and aggressive skin cancer, with a high tendency to both relapse and metastasize. 70-80% of MCCs carry the recently detected Merkel cell polyomavirus (MCV). MCV integrates clonally into the tumour genome and harbors specific mutations, which suggest a causal role in the tumorigenesis. Predispositional factors for MCC include advanced age, Caucasian race and immunocompromising states. The purpose of this study was to examine the etiological factors of MCC, particularly in the subgroups of immunodeficient and unusually young MCC patients. We also aimed to elucidate the biology and molecular mechanisms underlying the pathogenesis of the subgroup of tumours that do not present with MCV.

Methods: In the clinicopathological studies (I, IV and V), we combined clinical data derived from the Finnish Cancer Registry and patient records with biological features of MCC. All MCC diagnoses were confirmed histologically from the primary tumour paraffin blocks used as samples in the molecular analyses. MCV was analysed by quantitative PCR of the tumour samples. Expression of bcl-2 and RB proteins were examined by immunohistochemistry of tissue array blocks constructed from the primary tumour samples. Chromosomal aberrations were determined with array genomic hybridization, and the methylation of the RB1 promoter with methylation-specific multiplex ligation-dependent probe amplification technique. Standard statistical methods were employed to compare the findings between MCV-negative and positive tumour subgroups, and between patients younger and older than 50 years of age.

In the epidemiological studies (II and III) we utilized record linkage of the Finnish Cancer Registry and the National Prescription Register of the Social Security Institution. A case-control study with all MCC patients diagnosed in Finland was conducted to determine the role of autoimmune diseases as predictors of MCC. The effects of statin use on the incidence of MCC were studied in a cohort consisting of all statin users in Finland.
**Results:** The young MCC patients were frequently immunocompromised and MCV-positive. Bcl-2 expression did not vary according to MCV status and was associated with good survival in both groups. MCV-negative tumours were found to be chromosomally more unstable than MCV-positive tumours, and to harbor more frequent deletions of the chromosome 11p and the \( RB1 \) locus (7.7% vs 38%). The \( RB1 \) promoter was methylated in a distinctive pattern in MCC tumours, but did not vary according to MCV status. Autoimmune diseases in general (OR 1.63, 95%CI 1.19-2.22), and specifically rheumatoid and diabetic conditions, led to a predisposition for MCC. The incidence of MCC was significantly increased among young Finnish statin users (SIR 1.94, 95%CI 1.23-2.90). Paradoxically, the risk decreased significantly moving towards older age-groups of statin users.

**Conclusions:** Based on our results, autoimmune diseases and statin use play a part in the pathogenesis of MCC. The unusually young patients included in our material were also often immunocompromised. MCC should be kept in mind when dealing with skin manifestations of these subgroups. \( RB1 \) deletions and chromosomal instability are of importance in the pathogenesis the MCV-negative MCC tumours, whereas no differences were observed in bcl-2 expression between the subgroups. These findings provide further evidence that MCV infection defines subgroups of MCC.
INTRODUCTION

Merkel cell carcinoma is a rare cancer: the age-adjusted incidence of MCC in Finland is approximately 0.1 cases per 100 000 person-years (Kukko et al. 2012). It is even more lethal than melanoma with an overall five-year survival rate approximately 60% (Kukko et al. 2012; Agelli et al. 2010; Girschik et al. 2011). The mean age of MCC patients varies between 73 to 78 years (Savage et al. 1997; Dancey et al. 2006; Lyhne et al. 2011; Kukko et al. 2012). Patients under 50 years are rare, with a total representation of approximately 3% (Kukko et al. 2012; Kaae et al. 2010).

Immunosuppression is a clear contributing factor in MCC tumorigenesis. Immunocompromised MCC patients are younger than the average and their MCCs tend to act more aggressively (Penn et al. 1999; Brewer et al. 2012; Paulson et al. 2013). For example, renal transplant and HIV patients have a comparatively high age standardized risk of MCC (Koljonen et al. 2009b; Engels et al. 2002). Less is known of other forms of immunocompromising states.

The mechanisms of MCC pathogenesis have not yet been completely deciphered. The causal role of the Merkel cell polyomavirus (MCV) infection, which is detected in a majority of MCC tumours, is nowadays generally accepted. An apt immune system plays an important role in surveillance against viral infection as well as MCC progression. The infection raises MCV-specific immune responses, the intensity of which are associated with the prognosis of the disease. The effects of MCV infection on tumour biology and clinical features are under vigorous research (Sihto et al. 2009; Bergstrom 2008; Kanitakis, et al. 2006). Oncogenic pathways proven to be involved in the MCC pathogenesis include the RB- and p53-pathways (Kennedy et al. 1996; Houben et al. 2010a). Little is known of the molecular pathogenesis of MCC in the absence of MCV infection.
REVIEW OF THE LITERATURE

MERKEL CELLS AND THE IMMUNE SYSTEM

1. The Merkel cell as a part of normal skin

Merkel cells (MCs) constitute a normal yet scarce element of the basal layer of epidermis of skin and mucosa, forming dense patches in areas of specialized sensory perception such as the fingertips and the lips (Smith Jr. 1970). MCs typically contain keratin filaments, melanosomes and 80 nm dense-core neurosecretory granules that resemble those found, for example, in the adrenal medulla (Winkelmann et al. 1973). The surface of MCs features spine-like processes (Moll et al. 2005).

Figure 1: An electron microscopic image of a Merkel cell (MC) in the basal layer of rat epidermis bordering to the basal membrane (A). Spine-like processes, or pseudopodia (B), can be seen on the surface of the cell. The MC connects to keratinocytes (C) and makes a synaptic contact (D) with an afferent primary nerve ending to form the Merkel’s disk. Secretory granules are seen within the cytoplasm (E). Image from Dr. Jastrow’s Electron Microscopic Atlas by permission (http://www.drjastrow.de).
MCs are situated in close proximity of the terminal bulbs of afferent myelinated nerve fibres and hair follicles, and they are connected to keratinocytes by desmosomal junctions (Halata et al. 2003; Werling et al. 2011). They co-operate with the primary nerve endings forming the Merkel-cell-axon complex or Merkel’s disk, which works as a slow acting type I mechanoreceptor and is activated by steady skin indentation (Vos et al. 1991). MCs often lay close to Langerhans cells, and that has raised a hypothesis of MCs as part of the neuroimmunologic cutaneous system (Misery et al. 1996).

MC was first postulated to give rise to the Merkel cell carcinoma (MCC) based on the appearance of similar neuroendocrine granules in both MCs and MCC cells (Toker 1972). Factors for and against this hypothesis have later arisen. The hypothesis is supported by the fact that hyperplasia of Merkel cells is seen in chronic UV-exposure (Gould et al. 1985) and that cytokeratin 20 (CK-20) is expressed in both the carcinoma and MCs (Sadahira et al. 1987; Moll et al. 1986). However, mitoses have not been detected in MCs, and the tumour is by rule dermal, not epidermal (Moll et al. 1996b; Tang et al. 1979).

2. Immune responses against cancer cells

The evolution of normal cells to neoplasia requires the acquisition of capabilities that enable continuous tumour growth and progression. It is driven by genomic instability, sporadic mutations and chronic inflammation under the selective pressure of metabolic and immunologic challenges. The cells establish continuous growth by becoming immortal, escaping restrictive growth signals and in turn boosting proliferative signaling (Hanahan and Weinberg 2011). To support the increasing needs brought on by proliferation, the cells undergo major changes in their energy metabolism and induce a favourable tumour microenvironment by promoting angiogenesis and recruiting stromal cells, including endothelial cells, fibroblasts and cells of the immune system. The support network is maintained by the signaling changes of all of the involved cells and works in sync with the cancer cells to induce invasion and metastasis (Hanahan and Weinberg 2011).
The ability to evade immunosurveillance is crucial for tumour progression. The interaction between cancer cells and the immune system is often depicted with a model of ‘immunoediting’ consisting of three phases: the elimination, equilibrium and escape (Dunn et al. 2002). In the elimination phase, the invasive growth of a tumour causes tissue damage and inflammation attracting cells of the innate immunity. Foreign antigens processed by dendritic cells activate helper T-cell responses, which recruit tumour-specific cytotoxic T-cells (Koebel et al. 2007). Cytotoxic T-cells, macrophages and natural killer (NK) cells eliminate the malignant cells with the aid of an angiostatic and apoptotic chemokine response (Levy et al. 2011; Shankaran et al. 2001). If the elimination is incomplete, the escaped cancer cells go on into the ‘equilibrium phase’ where they continuously accumulate mutations under the selection pressure of the immune system (Dunn et al. 2002). In the ‘escape phase’, tumour cells that have acquired enough mutations finally escape the immune surveillance completely and start replicating without constrictions (Rangwala et al. 2011).

Malignant cells employ various mechanisms to evade the immune surveillance. They can lose the expression of major histocompatibility complex (MHC) class I molecules or certain tumour-specific antigens, or start expressing cell-surface proteins that inhibit immune responses (Chouaib et al. 2002; Poggi et al. 2006, shields JD 2010). Malignant cells also produce various immunosuppressive factors that modify the tumour microenvironment in the direction in which cells of both innate and adaptive immunity struggle to perform (Poggi et al. 2006; Yang L 2010). Regulatory T-cells (Tregs) are found infiltrating tumours and take part in controlling the inflammatory response. The tumour microenvironment can induce Treg dysregulation, which promotes tumour growth by curbing anti-tumour immunity (Whiteside 2012)

4. Secondary immunodeficiencies

Immunodeficiencies are classified as primary (congenital) or secondary (acquired). Primary immune deficiencies are caused by rare inherited gene defects that affect the cells of the immune system (Notarangelo 2010), and they are classified accordingly. Secondary
immune deficiencies are brought on by environmental factors, diseases, or iatrogenic measures, summarized in Figure 2 (Chinen et al. 2010). A common route in many of the mechanisms is the promotion of a state of chronic inflammation (Gudkov et al. 2010).

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tr>
<td>Immunodeficiency</td>
<td>An umbrella term depicting a wide spectrum of states in which the functions of the immune system are disrupted or even completely absent. Can be divided into primary and secondary. Patients with immunodeficiencies are called synonymously immunocompromised.</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>Generally refers to drug-induced immunodeficiencies in the cases of, for example, organ transplants and hematological malignancies</td>
</tr>
<tr>
<td>Immune deprived</td>
<td>Mostly refers to animals that have been genetically manipulated</td>
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Table 1: Definitions of terms depicting disturbances of the immune system. (Notarangelo 2010; Chinen, et al. 2010; Murpy 2012; Webber, et al. 2011) The terms are used with the above mentioned allocations throughout the dissertation.

Secondary immunodeficiencies lead to a predisposition for common infections, and occasionally even opportunistic infections can occur (Chinen et al. 2010). The patients often suffer from cancers of viral origin disproportionally (Harwood et al. 2000; Shibata et al. 1993). Immunosuppressed individuals are prone to a shorter life-expectancy, which might explain why the increased incidence of non-viral associated cancer first remained unnoticed (Dunn et al. 2002). Extensive epidemiological evidence also suggests that carcinomas of the lung, colon, bladder, ureter and endocrine origin, as well as malignant melanoma, are increased by immunosuppression (Birkeland et al. 1995; Pham et al. 1995; Penn 1996; Buell et al. 2005).
4.1. Environmental factors
Malnutrition with a lack of vitamins or minerals is the most common environmental cause of immunodeficiency (Black et al. 2008). Ultraviolet B radiation (UVB, 280-320nm) has both local and systemic immunomodulatory effects. UVB inhibits Langerhans cells from presenting antigens locally (de Gruijl 2008). It also induces systemically the apoptosis of T-cells, differentiation of Tregs and the release of cytokines such as IL-10 by keratinocytes and macrophages (Aubin 2003). The DNA damage and reactive oxygen species caused by UVB also contribute to the immunosuppression (Aubin 2003).

4.2. Diseases
The diseases leading to secondary immunodeficiencies are infectious, metabolic, hereditary or malignant (Chinen et al. 2010). Any prolonged infection can lead to chronic inflammation and the ensuing carcinogenic milieu (Gudkov et al. 2010; Nickoloff et al. 2005). The acquired immunodeficiency syndrome (AIDS) is an extreme case of immunosuppression caused by an infectious organism (Arron et al. 2011).

Autoimmune diseases (ADs) are caused by a mistargeted immune reaction to self, and lead to chronic inflammation and often metabolic disturbances. ADs are initiated by the combination of genetic predisposition and an activating environmental factor (Murpy 2012). Mutations in the HLA complex – with over 200 genes coding for leukocyte antigens, the MHC molecules – underlay ADs such as celiac disease and Type I diabetes mellitus (DMI) (Meresse et al. 2012; Undlien et al. 2001). ADs are associated with disturbances in restricting immune responses, including the function of regulatory T-cells and the cytokine spectrum. The chronic inflammatory stress is immunomodulating in itself and exerts carcinogenic effects via elevated free radicals and continuous cell proliferation (Nickoloff et al. 2005). Disturbances in the regulation of apoptosis are responsible for many of the immunologic changes (Nagata 2010; Rovere et al. 2000). Thus, disease-modifying antirheumatic drugs that induce apoptosis are used in the treatment of ADs (Murpy 2012).
Figure 2: Secondary immunodeficiencies summarized (Chinen, et al. 2010; Seppänen, et al. 2011). Usually, both innate and acquired immunity are affected. Quantitative changes include decreased levels of inflammatory cells and soluble inflammation mediators, while qualitative changes lead to the disturbed function of various components. (Seppänen, et al. 2011)
4.3. Iatrogenic factors

Iatrogenic causes of immunodeficiencies include radiotherapy, chemotherapy, surgery, and immunomodulating medication. Immunosuppressive therapy has been studied most widely in organ transplant recipients. Biologic therapies with monoclonal antibodies used in treatment of autoimmune conditions directly target proinflammatory mediators (Webber et al. 2011), while old chemical immunomodulatory drugs such as cyclosporine, corticosteroids, methotrexate and azathioprine also have direct mutagenic and photosensitizing properties (Rangwala et al. 2011). The extent of immunosuppression produced by many of the commonly used immunomodulating drugs such as corticosteroids and methotrexate are poorly known (Ducloux et al. 1998; Lott et al. 2010).

HMG-CoA inhibitors or statins have a beneficial effect on the inflammation caused by arterial disease independent of cholesterol levels (Goldstein et al. 2009; Palinski 2000). The idea of the anti-inflammatory-anticancer effects of statins was raised by observations of decreased cancer incidence in hospital studies, but no connections to either a preventive or predispositional direction have been detected in meta-analyses or record linkage studies (Pocobelli et al. 2008; Bjerre et al. 2001; Bonovas et al. 2006; Browning et al. 2007; Dale et al. 2006; Kuoppala et al. 2008).

The most studied immunomodulatory effect of statins is the downregulation of MHC class II expression, which leads to diminished T-cell activation and a shift from Th1 to the Th2 direction. This leads to increased B-cell activation (Kwak et al. 2000). Statins also act as pro-apoptotic agents by releasing nuclear antigens into the cells cytoplasm and inducing auto-antibodies (Blanco-Colio et al. 2002; Rovere, et al. 2000). In addition, the inhibition of cholesterol synthesis and the following disruption of lipid rafts, which are crucial for the activation of lymphocytes, might modulate the immune cells’ responses (Ehrenstein et al. 2005). Utilizing the immunomodulatory effects of statins in the treatment of various ADs and transplant rejection is under rigorous research (McCary et al. 2004; Mehra et al. 2002; Nie, et al. 2009; Rajpara et al. 2010).
4.4. Physiological changes
Newborn and old age constitute the immunocompromised periods of human life. The weakening of the immune system with advanced age is often referred to as ‘immunosenescence’, which affects both innate and adaptive immunity (Dorshkind et al. 2009). A gradual increase in the mediators of systemic chronic inflammation is observed (Gudkov et al. 2010). The production of hematopoietic growth factors regulating phagocytizing cells is diminished (Gomez et al. 2008). The production of oligoclonal CD8+ T cells and memory B-cells is increased, leading to a diminishing of naïve T and B-cells (Dorshkind, et al. 2009).

MERKEL CELL CARCINOMA

1. Epidemiology

Non-melanoma skin cancers (NMSCs) consist of primary cancers of the skin, including MCC, cutaneous lymphomas and adnexal tumours. The incidence of NMSC has increased substantially for several decades (Green 1992; Diepgen et al. 2002). Basal cell carcinoma (BCC) and squamocellular carcinoma (SCC) are the most common human cancers, and they are usually the ones meant when referring to NMSC. Common NMSCs are listed in Table 2, with their age-adjusted incidences in the Finnish population in 2011. The NMSCs in general have a low mortality (Veness 2008).

Epidemiological studies on MCC are often hindered by the fact that all NMSC are studied as one group. The rareness of the disease and the difficult differential diagnosis provide even more challenges (Heath et al. 2008; Koljonen et al. 2003). Until recently, most conclusions were based on clinical series, and the only available population-based studies were based on the Surveillance, Epidemiology, and End Results (SEER) Program managed by the U.S. National Cancer Institute (Agelli et al. 2003a). Precise diagnostics and wide-coverage registries have recently yielded studies, also from the Nordic countries,

<table>
<thead>
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<th>NMSC</th>
<th>Incidence ( /10^5 person-years)</th>
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<tr>
<td>Squamous cell carcinoma</td>
<td>96</td>
</tr>
<tr>
<td>Basal cell carcinoma</td>
<td>712</td>
</tr>
<tr>
<td>Mycosis fungoides</td>
<td>2.7</td>
</tr>
<tr>
<td>Merkel cell carcinoma</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 2: The incidences of common non-melanoma skin cancers (NMSC) per 1 00 000 person-years adjusted for age to the world standard population in Finland 2011. Mycosis fungoides is a form of cutaneous T-cell lymphoma. Data provided by the Finnish Cancer Registry (FCR).

MCC is approximately a hundred times rarer than melanoma (Landis et al. 1998), but more common than cutaneous T-cell lymphoma in the USA (Hodgson 2005). The age-adjusted incidence of MCC varies from 0.11 cases per 100 000 to 0.82 cases per 100 000 according to population and standardization (Kukko et al. 2012; Girschik et al. 2011; Lyhne et al. 2011; Kaae et al. 2010; Hussain et al. 2010). The lowest incidence has been recorded in Finland, where the age-adjusted (world standard) incidence was 0.11 per 100 000 person-years for men and 0.12 for women in 1989 to 2008 (Kukko et al. 2012). Adjusted to the European standard population, the incidences were 0.19 and 0.20, and adjusting to the US 2000 population 0.24 and 0.25. These are similar to the SEER-based age adjusted incidence in the USA (0.24 per 100 000 person years in 1986 to 1999) (Agelli et al. 2010). The highest age-adjusted incidences are recorded in Australia: 0.82 per 100 000 person-years in men and 0.63 in women standardized to the US standard population (Girschik et al. 2011).

A steep increase of MCCs has been recorded in recent decades in several populations. According to a SEER-based study, the incidence of MCC has risen 3-fold within 1986 to
This finding was further strengthened by a recent Danish study that reported a 5.4-fold rise in MCC incidence during 1986 to 2002 (Lyhne et al. 2011). The increase surpasses that of cutaneous melanoma (Hodgson 2005). The incidences of other NMSCs have also been increasing, but none as steeply as MCC (Hodgson 2005; Madan et al. 2010). In the elderly, the increase has been even steeper, from 0.39 per 100,000 person-years in 1986 to 1.76 in 2002 (Lyhne et al. 2011). The increase is partly explained by advances in diagnostic and coding procedures, as well as rising awareness among clinicians (Agelli et al. 2003a). The rise in the incidence since reached a plateau in the USA. (Agelli, et al. 2010) The incidence of MCC in Finland has remained quite stable since 1989 (Figure 4) (Kukko et al. 2012).

**Figure 3:** Incidence of MCC per 100,000 person-years according to calendar year at diagnosis in Finland, adjusted for age to the world standard population. Data extracted from the FCR. The first MCC case was reported in Finland in 1983. The first statin introduced to the Finnish market in 1987 was lovastatin (MSD Finland, personal communication). CK-20 was first used in MCC diagnostics in the year 2000 (personal communication, prof. Tom Böhling). ICD-O-3 coding (International Classification of Diseases for Oncology, Third Edition), which includes information on tumour morphology in addition to topography, was taken in to use in the FCR in 2008.
The mean age of MCC patients at diagnosis is approximately 75 years (Kukko et al. 2012; Kaae et al. 2010; Mills, et al. 2006; Reichgelt et al. 2011; Albores-Saavedra et al. 2010). The incidence increases with age, and patients under the age of 50-years are rare, constituting only fewer than 5% of all MCC patients (Kukko et al. 2012; Albores-Saavedra et al. 2010). MCC has been reported to affect children in rare cases (Kacker et al. 1992; Koksal et al. 2009; Schmid et al. 1992).

The vast majority of MCC patients are Caucasian. According to a SEER-based study, 93% of MCC patients were white, 1.3 % black and 4.3% belonged to other racial groups (Agelli et al. 2010). A slight male predominance has been observed in various population-based studies (Hussain et al. 2010; Albores-Saavedra et al. 2010; Girschik, et al. 2011). A slight female predominance was observed in a FCR-based study (Kukko et al. 2012). No differences between sexes were observed in Denmark (Lyhne et al. 2011; Kaae et al. 2010). It should be noted that the absolute number of female MCC patients is clearly higher than that of men because of the higher life-expectancy of females (Kukko et al. 2012).

In a SEER-based study, MCC patients presented with an increased number of other cancers in general for up to 1.22 times (95% CI 1.01-1.45) (Howard et al. 2006). The best-known connection of MCC is to other NMSC, but the standardized incidence ratios (SIRs) for malignant melanoma, Non-Hodgkin lymphomas, salivary gland tumours and biliary tract cancers are also elevated after MCC diagnosis (Howard et al. 2006). In a FCR-based study, the incidences of basalioma and chronic lymphocytic leukemia (CLL) were increased after the MCC diagnosis (Koljonen et al. 2010). A study based on a cohort of 335 MCC patients identified from the Israeli Cancer Registry concluded that the incidences of hematological malignancies, but not solid malignancies, were increased in MCC patients (Tadmor et al. 2012). Similar findings were made in a study of 351 MCC cases based on an Italian population: CLL was significantly more frequent, unlike solid tumours (Ascoli et al. 2011).
2. Clinical considerations

2.1. Clinical features
MCC tumours are presented typically as solitary, pink to purple dermal masses, which are painless but can grow rapidly to a substantial size with occasional ulceration (Figure 4) (Hitchcock et al. 1988; Yiengpruksawan et al. 1991). The tumours are most often located in areas of high UV exposure, with the head and neck area constituting approximately 50% of the cases (Kukko et al. 2012; Girschik et al. 2011; Agelli et al. 2010; Lawenda et al. 2001). MCC occurs rarely in the mucosa (~5% of cases) (Agelli et al. 2010). The larynx and the mouth are most often affected, and the tumours behave more aggressively than cutaneous MCCs (Agelli, et al 2010).

Figure 4: A typical ulcerating MCC tumour located in the pectoral/axillary region (courtesy of Dr. Virve Koljonen).

At the time of diagnosis, the disease is most often local (50-70% of cases), lymph nodes are affected in 24-30% of cases and distant sites in 5-10% (Kukko et al. 2012; Agelli et al. 2010; Girschik et al. 2011). Multiple non-radical excisions have often preceded admission to a tertiary center. Recurrence is common (Kukko et al. 2012). The most common sites of distant metastasis are distant lymph nodes, distant skin, lung, central nervous systems and
bone (Medina-Franco et al. 2001). Some patients present with a metastatic tumour without a known primary site (Kukko et al. 2012; Medina-Franco et al. 2001). The phenomenon can be explained either by spontaneous regression of the primary tumour, as recorded in several case series, or the excision of the primary with a benign clinical diagnosis (Connelly et al. 2000; Sais et al. 2002; Straka et al. 1997)

2.2. Diagnosis and staging

The histopathological diagnosis is based on the tumour morphology in hematoxylin and eosin (HE) staining and immunohistochemical stainings. In HE slides, the tumour cells are small, basophilic and monomorphic with sparse cytoplasm and nucleoli, resembling other small-round-blue cell tumours such as small cell lung cancer and neuroblastoma (Gould et al. 1985; Hitchcock et al. 1988; Tang et al. 1978; Pisick et al. 2003) MCC tumours most often grow into the subcutaneous fat. Tumour necrosis, high numbers of mitoses and

![Histological subtypes of MCC](image)

**Figure 5**: The histological subtypes of MCC. MCC is histologically divided into three subtypes (Gould, et al. 1985; Ratner, et al. 1993): from left to right: trabecular-, intermediate- and small-cell subtype. HE x100. The intermediate subtype is the most common. There is no correlation between the histologic subtype and the course of disease.
apoptosis are often seen (Bickle et al. 2004; Sidhu et al. 2005). Lymphatic invasion is typical for MCC, but perineural and vascular invasion can also occur (Mott et al. 2004; Kukko et al. 2010). Lymphocytes typically invade the tumour tissue and surround it (Bickle et al. 2004). MCC tumours express both cytokeratin and neuroendocrine markers, such as CK-20, chromogranin and synaptophysin (LeBoit et al.; Koljonen et al. 2005). A widely used, specific and sensitive diagnostic protocol is to combine CK -20 and thyroid transcription factor-1 (TTF-1) stainings (Ordonez 2000; Byrd-Gloster et al. 2000).

The consensus staging system by the American Joint Committee on Cancer (AJCC) has been the golden standard for MCC since 2010 (Table 3). Previously, the most applied system was the Memorial Sloan-Kettering Cancer Center classification (Allen et al. 1999). It was restricted to clinical investigation of possible nodal involvement, and as such did not serve prognostic estimates optimally (Lemos et al. 2007).

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<th>STAGE</th>
<th>lymph node</th>
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<th>systemic metastasis</th>
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<td>microscopic evaluation</td>
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<td>I</td>
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<td>II</td>
<td>primary lesion &gt; 2 cm</td>
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<td>III</td>
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<td>IV</td>
<td>systemic metastasis</td>
<td>+/-</td>
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Table 3: The American Joint Committee on Cancer staging system (AJCC) criteria for MCC.
2.3. Treatment and prognosis
No consensus has been reached in the treatment protocol of MCC. Radical excision in a tertiary center is the basis of treatment in all stages of disease, and a multidisciplinary approach is recommendable. Localized disease is treated with radical excision with clear margins, the satisfactory width of which is under constant debate (Allen et al. 2005; Poulsen et al. 2010; Finnigan et al. 2013). Recent studies have reported benefits of adjuvant radiotherapy, also in treating local disease with small tumours (Lok et al. 2012; Jouary et al. 2012; Ghadjar et al. 2011; Howle et al. 2012). Controversial findings in a SEER-based population suggest that the increase in survival seen with adjuvant radiation therapy is not disease-specific and might be a result of selection bias (Kim, et al. 2013).

Sentinel lymph node biopsy (SLNB) is recommended for all MCC patients by the National Comprehensive Cancer Network guidelines (NCCN 2010), if systemic spread has not been established. SLNB improves diagnostics and prognostic precision – approximately 30% of MCC cases that are estimated as local clinically present with microscopic nodal metastases in histologic examination (Howle et al. 2012; Lemos et al. 2010; Kouzmina et al. 2013).

Treatment of systemic MCC is mostly palliative and usually includes both local excision and radiotherapy. Postoperative chemotherapy has not been proven effective (Poulsen et al. 2006), despite trials with various chemical compounds (Balducci et al. 2010; Ely et al. 2008; Salavati et al. 2012; Wobser et al. 2009; Shah et al. 2009). The most frequently administered chemotherapeutic agents are those used in other neuroendocrine carcinomas, most commonly polychemotherapy with cisplatin, etoposide and doxorubicin (Tai et al. 2010; Gessner et al. 2011).

Tumour size is invariably recognized as the most consistent prognostic factor, and is as such incorporated into the staging system (Koljonen et al. 2003; Allen et al. 2005). Similarly, nodal involvement and distant metastasis indicate poor prognosis (Kukko et al. 2012; Girschik et al. 2011; Kaae et al. 2010). Tumour location in the upper limb is associated with a better prognosis compared to other locations (Agelli et al. 2010; Smith et
The effect of age to disease-specific survival is controversial (Kukko et al. 2012; Agelli et al. 2010). Several studies have reported better survival in male than female patients (Kukko et al. 2012; Agelli et al. 2010; Reichgelt et al. 2011). Immunosuppression impairs prognosis, but the occurrence of other malignancies has not been associated with survival (Girschik et al. 2011; Guler-Nizam et al. 2009).

Tumour thickness is not independently associated with survival (Goldberg et al. 2007; Izikson et al. 2012). Infiltration to subcutaneous fat, diffuse growth pattern, lymphatic invasion, dense vascularization, tumour mast cell count, and heavy infiltration of lymphocytes are variably suggested as histopathological features indicating poor prognosis (Mott et al. 2004; Llombart et al. 2005; Andea et al. 2008; Ng et al. 2008; Beer et al. 2008).

3. Etiology

3.1. MCV infection
MCV is a typical non-enveloped polyomavirus containing a circular double stranded DNA genome of approximately 5kB packed in a 45-50 nm capsid. MCV expresses the small T-antigen (ST), the large T-antigen (LT), the 57kT-antigen and capsid proteins VP1, VP2 and VP3 (Shuda et al. 2008). MCV was discovered in 1998 by Feng and colleagues (Feng et al. 2008). They detected a fusion transcript between a novel virus T antigen and a human receptor tyrosine phosphatase by using digital transcriptome subtraction in ten MCC tumours. MCV has the highest homology with the murine polyomaviruses and less similarities to the known human polyomaviruses (BK or JC viruses) and to the simian virus 40 (SV40) (Feng et al. 2008). MCV sequences were found in 8 of 10 MCC tumours, but in only in 4 of 25 control skin tissue samples.

A significant proportion of the human population has encountered MCV (Kantola et al. 2009). It was found in 71% of non-tumourous skin samples of MCC patients, and in 62-80% of skin swabs from healthy persons (Foulongne et al. 2010a; Foulongne et al. 2010b; Wieland et al. 2009). The IgG seroprevalence against MCV virus-like particles (VLPs) constructed from viral capsid proteins is 50% in children under 15 years, and up to 80% in
people older than 50 years (Tolstov et al. 2009). Several findings indicate that MCV does not just secondarily infect tumour cells. IARC released a consensus statement declaring the direct causal nature of MCV in 2012 (WHO, International Agency for Research on Cancer 2012). General criteria for defining a direct carcinogenic role for an infection in cancer are listed in Table 4, with correlates to MCV infection.

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<td>A</td>
<td>There is plausible epidemiological evidence that the viral agent is a risk factor for the malignancy</td>
<td>(Sihto, et al. 2009; Garneski, et al. 2009; Bhatia, et al. 2010a)</td>
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<td>B</td>
<td>Nucleic acids of the virus persist in each tumour cell</td>
<td>(Shuda, et al. 2008; Feng, et al. 2008)</td>
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<td>C</td>
<td>The virus becomes immortal after target cells growth in <em>vitro</em></td>
<td>(Shuda, et al. 2008)</td>
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<tr>
<td>D</td>
<td>Viral oncogenes are expressed, and they interact with cellular proteins in a way that leads to disruption of cell-cycle control and inhibition of apoptosis. When cell lines are transfected with the viral genome or parts of it, their proliferation is stimulated</td>
<td>(Shuda, et al. 2008; Shuda, et al. 2011; Houben, et al. 2012)</td>
</tr>
<tr>
<td>E</td>
<td>The maintenance of the malignant phenotype and proliferation of tumour cells can be demonstrated to be dependent on the persistence of viral DNA</td>
<td>(Houben, et al. 2010b)</td>
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*Table 4:* The general criteria for a direct carcinogenic role of virus infection in the pathogenesis of a malignancy. References of MCV and MCC regarding each criterion are listed on the right (WHO, International Agency for Research on Cancer 2012).
The following findings support the causal role of MCV in MCC pathogenesis:

1. MCV DNA integrates into the tumour genome in a monoclonal pattern, suggesting that the virus infection and genomic integration take place prior to clonal expansion of the tumour cells (Feng et al. 2008). The same monoclonal pattern is seen in the respective metastases of MCC tumours (Feng et al. 2008).

2. The copy numbers of MCV DNA are considerably higher in MCC tumour tissues than in control samples from non-tumourous tissues or healthy controls (Garneski et al. 2009). MCC derived MCV genomes undergo specific truncating mutations of the LT that render the virus incapable of replication and thus protect cancer cell survival (Shuda et al. 2008). These mutations are not detected in viral genomes of nonmalignant origin (Shuda et al. 2008; Schmitt et al. 2012).

3. The role of LT as the primary viral oncogene is supported by the finding that unlike antibodies against VP1 and other capsid proteins, the antibodies against LT are specific for MCC patients. Antibodies against LT are found in 40.5% of MCC patients and only in 0.9% of the healthy controls (Paulson et al. 2010). Furthermore, the levels of LT antibodies increase in MCC patients whose cancer progresses locally or metastasizes.

4. MCC derived MCV-positive tumour cells cultured in vitro undergo growth arrest after LT-knockdown, and consequently require the expression viral T-antigens to maintain their growth (Houben et al. 2010b).

MCV integrates into random sites of the tumour genome (Feng et al. 2008; Sastre-Garau et al. 2009). Copy numbers of the MCV genome vary from less than one viral DNA copy per 10 000 tumour cells to ten viral DNA copies per a single tumour cell (Sihto et al. 2009; Garneski et al. 2009). Proportions of MCV-positive tumours vary according to the geographic location. In Europe the proportion is higher (up to 85%) than in the USA (69%) or in Australia, which has the smallest number of virus positive MCCs (21-24%) (Sihto et al. 2009; Garneski et al. 2009; Becker et al. 2009). MCV strains in Europe are conserved and closely resemble the MCC 339 strain in the USA, pointing to genetic stability (Touze et al. 2009).
The widespread expression of MCV in a healthy population suggests a simple, day-to-day transmission mechanism. MCV-positive tumours are often located in the extremities, which suggests transmission through physical contact (Sihto et al. 2009). Fecal-oral transmission has been suspected on the basis of high portions of MCV DNA in the saliva of healthy subjects (Loyo et al. 2010). Another possible route might be via the respiratory track, since MCV sequences are found in tonsillar tissue, respiratory secretions and mouthwash (Kantola et al. 2009). The urinary tract is employed in transmission by some polyomaviruses, but in the case of MCV this seems unlikely since no MCV DNA is found in the prostate and surrounding tissues (Bluemn et al. 2009).

The detection and copy number count of MCV are usually determined by protocols that utilize quantitative polymerase chain reaction (PCR) with primers set to amplify different viral coding regions, most often the LT or VP1 coding regions. Shuda and colleagues developed a monoclonal antibody (CM2B4) that recognizes MCV LT (Shuda et al. 2009). It shows MCV expression in nuclei of cells that have an average of 5.2 (range 0.8–14.3) LT DNA copies per cell when quantified by PCR. The antibody is commercially available. Immunohistochemistry for MCV has value in differential diagnostics between MCC and other poorly differentiated neuroendocrine carcinomas presenting in the skin (Busam et al. 2009). Increasingly sensitive detection methods have since been developed. Rodig and colleagues recently reported that traces of MCV DNA were detected in all 60 specimens of a MCC series when a wider range of PCR primers was used (Rodig et al. 2012).

### 3.2. Immunodefiencies

Immunosuppressed patients are at a high risk of developing virus-associated cancers in general (Zur Hausen 2009). Increased incidence of MCC in immunosuppressed individuals was first reported in case reports and organ transplant registry studies, and the connection raised a suspicion of a possible viral agent (Penn et al. 1999; Koljonen et al. 2009b; Tadmor et al. 2012; Feng et al. 2008; Buell et al. 2002). Copious case reports describing the spontaneous regression of MCC tumours after biopsy or cessation of immunosuppression also pointed to the importance of immune functions in the pathogenesis of the carcinoma (Connelly et al. 2000; Straka et al. 1997; Castano et al. 2012; Val-Bernal et al. 2011; Wooff et al. 2010; Turk et al. 2009). According to
retrospective clinical studies, approximately six to eight percent of MCC patients have a history of some form of an immunodeficiency (Girschik et al. 2011; Heath et al. 2008).

In a Finnish record linkage study, the incidence of MCC among renal transplant patients undergoing immunosuppressive medical treatments was 66-times higher than among the normal population (95% CI 14-194, p< 0.001) (Koljonen et al. 2009b). The incidence of MCC is also increased after the diagnosis of lymphoid malignancies, specifically CLL and non-Hodgkin lymphoma (NHL) (Kaae et al. 2010; Heath et al. 2008; Koljonen et al. 2009a). A connection exists in the opposite direction as well – MCC patients have an increased risk of developing CLL (Howard et al. 2006; Koljonen et al. 2010). An association between MCV infection and CLL has been established, but causality has not been proven (Pantulu et al. 2010; Tolstov et al. 2010). AIDS patients are in up to an 11 times greater risk of developing MCC than the average, but the absolute number of MCC patients with HIV infection or AIDS is low (Engels et al. 2002; Kaae et al. 2010).

Only two epidemiological studies have addressed the role of ADs as risk factors for MCC separately from other NMSCs. Hemminki and colleagues found an increased risk of MCC among patients with inflammatory bowel disease (SIR 4.4, 95%CI 1.8-7.7) and ankylosing spondylitis (SIR 16, 95%CI 3.0-46) (Hemminki et al. 2012). Rheumatoid arthritis was linked to MCC in the SEER-based case-control study of elderly adults by Lanoy and Engels (OR 1.4, 95%CI 1.1-1.8) (Lanoy et al. 2010). Patients with autoimmune diseases often receive immunosuppressive therapy, the effects of which are hard to set apart from the underlying condition. Some case reports have reported partial regression of MCC after the cessation of immunosuppressive therapy used in the treatment of an AD (Friedlaender et al. 2002; Muirhead et al. 2007). Two case reports have described the development of MCC after launching therapy with TNF-α inhibitors, but no large scale studies on biological disease-modifying antirheumatic have been conducted (Krishna et al. 2011; Linn-Rasker et al. 2012).

Immunodeficiencies not only lead to a predisposition for MCC, but also affect the clinical course of the cancer. Immunodeficient patients are significantly younger than usual, with
the average age of less than 60 years at diagnosis (Penn et al. 1999; Koljonen et al. 2009b; Engels et al. 2002; Buell et al. 2002). Moreover, the survival of patients with systemic drug-induced immunosuppression or concurrent CLL is decreased (Brewer et al. 2012; Paulson et al. 2013).

The epidemiological link between immunosuppression and MCC is quite clear, but the mechanisms and disruptions in the immune functions allowing for aggressive tumour growth in immunocompromised MCC patients are poorly known. Furthermore, there is little to no information on the effects of particular immunosuppressive medications on the incidence or behaviour of MCC.

3.3. Ultraviolet radiation

MCC patients are mostly Caucasians with fair skin (Albores-Saavedra et al. 2010; Agelli et al. 2003). 82-90% of MCC tumours appear on areas of skin that are frequently exposed to sunlight (Medina-Franco et al. 2001; Allen et al. 2005). The tumours are most often located on the left side of the body which is typical for UVR induced carcinomas (Paulson et al. 2011a; Koljonen et al. 2013). In addition, the incidence of MCC is comparatively high at equatorial latitudes (Girschik et al. 2011; Hodgson 2005).

The mutagenic effects of UVR are mostly associated with UVB radiation (290-320nm) (Afaq et al. 2005). For every unit of increase in the UVB solar index, the logarithms of the incidence rate of MCC increase by 0.00021 (Miller et al. 1999). MCC genomes harbor C-to T transitions and UVB-specific mutations of TP53 and Ha-ras genes similar to other UVR induced skin cancers (Van Gele et al. 2000; Popp et al. 2002). According to the current view, the carcinogenic effects of UVR are mediated principally via immunomodulation (Oberyszyn 2008). UVR is one of the most important etiological factors of NMSC, especially those with viral association (Arron et al. 2011). In a recent experimental study, MCV ST mRNA levels increased following in vivo UV-radiation, which points to delicate interplay between MCV infection and UVR exposure in the pathogenesis of MCC (Mogha et al. 2010).
4. Tumour biology

4.1. Viral oncogenesis
LT expression is crucial to the malignant progression of MCV-positive MCC \textit{in vitro}. MCC-specific mutations of the LT are equally crucial in preventing viral reactivation to ensure host cell survival and deactivating growth inhibitory signals (Shuda et al. 2008; Houben et al. 2010b; Cheng et al. 2013). The large tumour antigens of polyomaviruses are known to bind p53 and RB, which suppress tumour cell cycle (Ahuja et al. 2005). Mutations truncating MCV LT do not affect its ability to bind RB, but the ability to bind p53 is lost (Shuda et al. 2008).

LT binding to RB leads not only to the sequestration of RB, but also to the increased expression of survivin (Arora et al. 2012). Survivin is an anti-apoptotic protein strongly expressed in embryonic tissues and several cancers. High survivin expression in MCC is linked to poor prognosis (Kim et al. 2008). Administration of exogenous LT with an intact RB binding domain leads to the up-regulation of the G1 to S proteins, including the anti-apoptotic survivin, E2F1 transcription factor and cyclin E (Arora et al. 2012). MCV-positive tumours express survivin seven-fold more than MCV-negative tumours, and a survivin inhibitor has been reported to halt the growth of MCV-positive MCC xenograft tumours (Arora et al. 2012).

The LT suppresses natural responses to UVR-induced DNA damage (Demetriou et al. 2012). Ectopically expressed truncated LT reduced the expression of p53, p21 and the DNA damage recognition protein XPC in a line of MCC cells (Demetriou et al. 2012). Since truncating mutations lead to the loss of p53 binding ability, the mechanisms behind the p53 down-regulation remain unclear (Shuda et al. 2008).

MCV ST transforms rodent fibroblasts to anchorage and contact-independent growth \textit{in vitro}, and promotes serum-free proliferation of human cells (Shuda et al. 2011). Shuda and colleagues reported that the inhibition of ST causes arrest in the MCC cell cycle, while another recent \textit{in vitro} study maintains that ST expression is not required for MCC cell
survival (Shuda et al. 2011; Angermeyer et al. 2013). ST enhances the LT-mediated viral replication through interactions with protein phosphatase 2A (Feng et al. 2011; Kwun et al. 2009). It also acts downstream in the mammalian target of rapamycin (mTOR) signaling pathway in order to maintain the hyperphosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (Shuda et al. 2011).

4.2. The hit and run hypothesis
MCC has historically been divided in two clinically distinct subgroups (Leonard et al. 1993). Viral status is now postulated to be the distinguishing factor between these subgroups. High copy numbers of MCV DNA in MCC tumours are linked to young age at diagnosis of MCC and improved survival (Bhatia, et al. 2010a). MCV-negativity or low viral copy number and lack of LT expression are respectively associated with frequent metastasis and poor survival (Sihto et al. 2009; Andres et al. 2009). Some studies have reported contradictory results, or found no significant differences in the course of disease or survival (Becker et al. 2009; Handschel et al. 2010; Schrama et al. 2011).

VP1-antibody levels of healthy individuals correlate with the level of MCV DNA found on the skin, but exceptionally high anti-VP1 antibody levels occur in MCC patients even when their tumours present with less than one viral genome per cell (Touze et al. 2011). Since VP1 antibody levels do not correlate with the viral copy number in MCC patients, Houben and colleagues suggested a model in which MCV is a causal factor of MCC, including when the MCV genome cannot be detected (Houben et al. 2010b). This ‘hit-and-run hypothesis’ suggests that integrated MCV DNA can be eventually lost, while the oncogenic phenotype is retained. Infected cells would undergo mutations enabling tumour progression even without LT expression. These mutations would then lead to the loss of MCV DNA and finally to the differences seen in biological behaviour between MCV-positive and negative MCC tumours. The model is known from adenovirus-transformed hamster cells (Houben et al. 2010b).
4.3. Host defence against MCV and immune evasion
MCC patients show high levels of neutralizing anti-VP1 antibodies even when the tumour cells do not express detectable amounts of the capsid protein (Pastrana et al. 2009). It is possible that the strong humoral response against VP1 could be primed by a highly immunogenic MCV primary infection instead of depending on continuous expression of the protein (Pastrana et al. 2009). High levels of antibodies against VP1 improve the prognosis of MCC (Touze et al. 2011). Thus, the use of MCV VLP particles as vaccine against MCC has been suggested (Pastrana et al. 2009).

Unlike the capsid proteins, the T-antigen oncoproteins are continuously expressed and required for tumour growth (Houben et al. 2010b). IgG antibodies against ST and LT are relatively specific to MCC: they are found in 40.5% of MCC patients but only in 0.9% of the healthy controls (Paulson et al. 2010). The levels of LT and ST antibodies correlate to tumour mass, and rise at the event of spread or metastasis of MCC (Paulson et al. 2010).

MCV-specific T-cell responses detected in the peripheral blood of MCC patients are characterized by CD4+ helper cells, which react to a broad range of peptides derived from viral capsid and oncoproteins (Iyer et al. 2011). MCV-specific CD4+ helper T-cell responses can be induced with VLPs in both MCV-seropositive and negative healthy volunteers, but the responses are stronger in MCV-positive patients (Kumar et al. 2011). Surveillance for MCV infection is not mediated only by humoral immunity and CD4+ Th1-cells, but also by the cell mediated immunity. MCV specific CD8+ T-lymphocytes are found in the peripheral blood in over half of MCV-positive MCC patients (Afaniesiev et al. 2013a). The levels of circulating MCV specific CD8+ T-cells increase with disease progression and decrease with successful MCC treatment (Afaniesiev et al. 2013a).

MCV-positive tumours contain significantly increased numbers of tumour infiltrating CD8+ and CD3+ lymphocytes, NK-cells, macrophages and Tregs (FoxP3+) compared to virus negative tumours (Harms et al 2012, Sihto et al. 2012). MCV-specific CD8+ and CD4+ lymphocytes can be isolated from MCV-positive MCC tumours, but not from MCV-negative tumours (Iyer et al. 2011). The number of CD8+ lymphocytes correlates with a favourable
prognosis of MCC (Paulson et al 2011, Harms et al. 2012, Sihto et al. 2012). High CD8+/CD4+ and FoxP3+/CD4+ ratios as well as CD3+ numbers are also associated with good survival (Sihto et al. 2012). The effect on survival is not restricted to MCV-positive tumours (Sihto et al. 2012). High numbers of CD3+ TILs are linked to less frequent metastasis, but not to proliferation markers such as Ki-67. This suggests that the survival effect of TILs could be brought on by hindering metastasis rather than tumour proliferation (Sihto et al. 2012). The functions of Tregs in MCC remain unspecified. FoxP3+ cells have been identified in the microenvironment of one MCC tumour together with MCV-specific cytokine IL10 and IFN-γ secretion, which has been linked to regulatory function in chronic antigen exposure (Iyer et al. 2011). In vitro administration of interleukin-2 and interleukin-15 induced the proliferation of FoxP3+ Tregs (Dowlatshahi et al. 2013).

The immune system is actively involved in the pathogenesis of MCC (Figure 6). Even though high levels of neutralizing antibodies against VP1 and T-antigens occur in MCC patients and MCV-specific TILS are found in the tumours, they do not prevent MCC tumorigenesis or the spread of disease. Local and systemic immunodefiencies diminish the immune responses against MCV and leave the patients susceptible for the immune escape of the transformed cells. The tumour induced microenvironment plays its part in promoting immunoevasion to allow for disease progression.

MCC tumour cells have been hypothesized to employ particular mechanisms to evade the tumour surveillance by TILs. MCV-specific CD8+ T-cells are enriched among TILs of MCC tumours in comparison to leukocytes of the blood implying unhindered T-cell trafficking into the tumour (Iyer et al. 2011). However, the loss of vascular E-selectin expression, an important factor in T-cell entry to the skin, correlates with poor intratumoral CD8+ infiltration and prognosis in MCC (Afanasiev et al. 2013). The chronic exposure to T-antigens may lead to an exhaust type CD8+ phenotype with poor effector function (Iyer et al. 2011; Frebel et al. 2010; Dowlatshahi et al. 2013). Decreased activity of TILs in MCC is seen as decreased expression of costimulatory signal molecules CD69 and CD25, as well as expression of the T-cell exhaustion markers PD-1 and Tim-3 (Dowlatshahi et al. 2013, Afanisiev et al. 2013b). The expression of PD-L1, a lymphocyte inhibitor receptor ligand co-acting with PD-1 in various tumour microenvironments, correlates with the number of
CD8+ lymphocytes in MCC tumours (Afanisiev et al. 2013a). These findings suggest that restricting T-cell entry into the tumour and their functional properties might be considerable and even targetable forms of immunoevasion in MCC.

Figure 6: MCV infection and the immune system in the pathogenesis of MCC (Bhatia, et al. 2011) © Springer Science and Business Media, by premission. MCV infection occurs in childhood and leads to both humoral and cellular immune responses (Tolstov et al. 2009; Pastrana et al. 2009). The viral genome undergoes mutations that truncate the large T-antigen, which is later expressed persistently and has an important role in MCC oncogenesis (Shuda et al. 2008; Houben et al. 2012). Immunodeficiencies may lead to disturbances in surveillance against virus infection and malignant cells. Disease progression can generally be monitored via immune biomarkers such as anti-T Ag antibody and CD8+ TIL levels (Paulson et al. 2010; Paulson et al. 2011b).
The MCV genome partakes in controlling the immune responses against MCC cells. LT and ST-antigens downregulate epithelial and MCC cells’ Toll-like receptor 9 gene expression, which is crucial for innate immune reaction against foreign DNA (Shahzad et al. 2013). MCV also encodes microRNA MCV-mir-M1-5p, which is predicted to target genes that may be involved in immune-related pathways (Lee et al. 2011).

### 4.4. Molecular pathogenesis
Most of the genomic aberrations of MCC tumours are gains of whole chromosomes or complete chromosome arms (Larramendy et al. 2004; Harle et al. 1996; Gancberg et al. 2000; Van Gele et al. 1998). Gains are approximately twice as common as losses (Larramendy et al. 2004). High-grade amplifications are rare (21%). The most frequent chromosomal aberrations of MCC include the trisomy of chromosomes 6, 12 and X, gains in 1q, 1p, 3q, 5p, 19q and p, as well as losses in 3p, 1p, 4q and 13q (Larramendy et al. 2004; Van Gele et al. 1998). Large tumours and MCV-negative tumours harbor more numerous changes (Larramendy et al. 2004; Van Gele et al. 1998; Paulson et al. 2009). Low numbers of genomic changes are associated with a good prognosis (Paulson et al. 2009). Recurrent sites of MCV integration have not been observed, nor alterations of cellular genes located near the viral sequences (Sastre-Garau et al. 2009).

The molecular basis of MCC in the absence of MCV infection is still poorly known. MCV-negative tumours and tumours with low viral copy numbers are linked to the loss of retinoblastoma protein (RB) expression (Sihto et al. 2011; Bhatia et al. 2010b), increased c-KIT expression (Waltari et al. 2011), increased p53 expression (Bhatia et al. 2010a; Waltari et al. 2011), and TP53 mutations in a subset.

**Cell cycle regulation by RB and p53 in MCC**
Considering the importance of the RB sequestering in viral oncogenesis, other means of impacting the RB function have been under examination in MCV-negative tumours. *RB1* is a well characterized tumour suppressor gene that codes the RB protein. RB regulates the transmission from G1- to S phase in the cell cycle by inhibiting the E2F transcription factor. Cyclin/cyclin dependent kinase complexes phosphorylate the RB and detach it from the
RB-E2F complex, which commences the cell cycle. Several inhibitory proteins such as p16, p21 and p27 oppose the cell cycle entry (Sherr et al. 2002).

Sihto and colleagues studied retinoblastoma expression in MCV-positive and negative tumours by immunohistochemistry and observed that all MCV associated MCC tumours expressed RB, whereas 87% of LT negative tumours were also RB negative (Sihto et al. 2011). LT and RB expression were associated with a better outcome. There was no connection between LT expression and expression of phosphorylated RB, indicating that phosphorylation is not required to down regulate RB in MCV-positive tumours. Bhatia and colleagues reached similar results in comparing RB immunohistochemistry and MCV status, whereas Houben and colleagues found that all tumours expressed RB despite viral status (Houben et al. 2010a; Bhatia et al. 2010a). Comparison of the gene expression profiles of MCC tumours by transcriptome analysis revealed 2.4 times lower $RB1$ expression in MCV-negative tumours (Harms et al. 2012). All of the MCV-positive tumours expressed RB. In immunohistochemical validation, whereas only less than half of the negative tumours were RB positive.

Deletions of $RB1$ locus have been described widely (Larramendy et al. 2004; Van Gele et al. 1998; Paulson et al. 2009). Paulson and colleagues detected a narrow region deletion of 13q14.11-13q21.33 which contains less than 100 anointed genes, including $RB1$ (Paulson et al. 2009). Six out of 23 tumours presented with the deletion, four of these being virus negative. It seems that loss of RB is an integral part of MCC pathogenesis, either by inactivation by LT or by other mechanisms in MCV-negative tumours. A substantial part of MCV-negative tumours do express RB, and the mechanisms for RB dysregulation in these tumours remain unspecified. For example, silencing the $RB1$ by the promoter hypermethylation has not been studied in MCC, although it plays an important part in the pathogenesis of several malignancies (Sakai et al. 1991; Yoshino et al. 2007; Nakamura et al. 2001; Malekzadeh et al. 2009).

p53 is activated upon a multitude of different stressors including DNA damage, and it acts as a transcription factor regulating genes that are involved in controlling the cell cycle,
apoptosis and DNA repair (Horn et al. 2007). Even though the LT loses its p53 binding domain when truncated, it may target p53 via more indirect routes in MCV-positive MCCs (Demetriou et al. 2012; Shuda et al. 2008). However, LT knock-down does not lead to increased p53 activation in vitro (Houben et al. 2013). The p53 wild type has a short half-life and is not usually detected in immunohistochemistry unless mutated or stabilized via interactions with intracellular proteins. p53 expression in immunohistochemistry is rare in LT negative MCCs but even rarer in MCV-positive MCCs, suggesting low rates of mutations (Sihto et al. 2011; Kennedy et al. 1996; Feinmesser et al. 1999; Koljonen et al. 2006). TP53 mutations only occur in the LT- negative MCC tumours (Sihto et al. 2011). These mutations are of the signature UVB type, which is in line with the role of UVR in MCC etiology (Popp et al. 2002).

p53 induces the expression of p21 and p16\(^{INK4a}\), which are cyclin-dependent kinase inhibitors and prevent the phosphorylation of RB. p14\(^{ARF}\) is another p53 regulator, which acts by preventing the degradation of p53 (Horn et al. 2007). Both p16\(^{INK4a}\) and p14\(^{ARF}\) are down regulated by methylation: in MCC, silencing by methylation has been reported in in eight out of 19 MCC in p14ARF promoter, but only in one sample in the p16INK4a promoter (Lassacher et al. 2008). p53 could in this sense be inactivated by inhibition of p14\(^{ARF}\) expression. The hypothesis was tested by treating MCC xenografts with farnesylation inhibitors, which lead to p53 depression and induction of apoptosis (Jansen et al. 1999).

### Regulation of apoptosis by bcl-2 in MCC

The bcl-2 protein family act in opposing the intrinsic pathway of apoptosis, which is brought on by crucial changes in mitochondrial functions (Leverkus et al. 2008). Increased bcl-2 expression is a common way for tumour cells to evade apoptosis, and it is crucial in the UVR mediated photocarcinogenesis of skin cancers (Nys et al. 2012). It also mediates the apoptotic and immunosuppressive effects of various immunomodulatory drugs such as corticosteroids and statins (Lynch et al. 2010; Spampanato et al. 2012; Xu et al. 2008). Bcl-2 has been reported to be expressed in approximately two thirds of MCC tumours in two small scale studies (Kennedy et al. 1996; Feinmesser et al. 1999). No connection to aggressive behaviour of the disease was noted. Although MCC is radiation sensitive, bcl-2
expression indicates that some cells might be resistant to apoptosis inducing therapies, including radiation and chemotherapy (Feinmesser et al. 1999). Bcl-2 inhibition in xenograft mice by antisense oligonucleotides resulted in decreases in tumour size (Schlagbauer-Wadl et al. 2000). No significant effects were noted when the oligonucleotides were tested in the phase II trial on patients with advanced MCC (Shah et al. 2009).

The ratio between bcl-2 and another member of the bcl-2 family, the proapoptotic bcl-2 homolog bax, has a great impact on the overall regulatory effect (Oltvai et al. 1993). Bax is widely expressed in MCC (Feinmesser, et al. 1999). p53 induces bax expression directly, and suppresses Bcl-2 expression (Miyashita et al. 1994; Miyashita et al. 1995).
AIMS OF THE STUDY

I  To study the etiological features and clinical characteristics of Merkel cell carcinoma (MCC) presenting at an atypically young age of 50 years or less.

II To assess the incidence of autoimmune diseases (ADs) among Finnish MCC patients, and to evaluate whether ADs could lead to a predisposition for MCC.

III To study the effects of the widespread use of HMG CoA reductase inhibitors on the incidence of MCC in Finland, and to assess whether their use increases the risk of MCC in the population.

IV To evaluate the usefulness of the immunohistochemically detected bcl-2 expression in predicting the prognosis of MCC. We also aimed to find out if the expression of this anti-apoptotic protein varies in MCC tumours according to exposure to MCV infection or immunomodulating factors such as UVR or aging.

V To shed light to the molecular pathogenesis of MCC in the absence of MCV infection by studying chromosomal aberrations distinctive to MCV-negative tumours. We specifically aimed to find explanations to the frequent losses of RB expression in MCV-negative tumours by concentrating on the possible losses of the RB1 locus and by defining the promoter methylation pattern of the RB1 gene.
PATIENTS AND METHODS

An institutional Ethics Committee approved the study protocols. The permission to identify MCC patients from the FCR was obtained from the National Institute for Health and Welfare. Permission to analyse the tissue samples was obtained from the National Agency for Medicolegal Affairs. The National Social Insurance Institution (SII) provided permission for the data on reimbursements of medical expenses used in studies II and III.

CLINICOPATHOLOGICAL STUDIES I, IV AND V

1. Tumour samples and clinical data

We began our search for MCC tumours by identifying patients diagnosed with MCC during 1979-2004 in Finland from the FCR and Helsinki University Hospital records. In addition, MCC was searched with synonymous diagnostic names, such as small cell carcinoma of the skin. Available formalin-fixed, paraffin-embedded tissue blocks matched to the notified MCC cases were collected from the pathology archives of various pathology laboratories around Finland.

We confirmed all of the MCC diagnoses histologically before accepting tumour samples for further studies. This included staining with H&E and immunohistochemistry for CK-20 (DakoCytomation, Glostrup, Denmark) and TTF-1 (Novocastra, Balliol Business Park West, Benton Lane, Newcastle Upon Tyne, UK). The histological MCC subtypes and tumour diameters were assessed from the HE slides. If the preparations only covered partial tumours, information on the tumour diameter was retrieved from the hospital case records.

The clinical data used in studies I, IV and V was collected for the histologically ascertained MCC cases. Noteworthy data included age, sex, medical history, size and location of the primary tumour, disease status at presentation (nodal and systemic metastasis), date and
method of initial surgery, radiation therapy, adjuvant chemotherapy and recurrence. The
date and cause of death were derived from the FCR through linkage to the population
register. The overall survival was counted from date of MCC diagnosis to the date of death
from any cause or the end of follow-up. MCC-specific survival was counted from diagnosis
to death from MCC or the end of follow up.

2. Detection of Merkel cell polyomavirus DNA

Information on MCV expression was utilized in studies I, IV and V. MCV detection from the
tumour paraffin blocks had been carried out in our previous projects and is described in
detail elsewhere (Sihto et al. 2009). In summary, the process included DNA extraction and
the quantitation of MCV DNA with real-time PCR, expressed as a ratio of MCV DNA
genome to a reference gene DNA (protein tyrosine phosphatase gamma receptor gene).
Whenever the MCV DNA copy number per reference gene was greater than 0.1, the
sample was considered MCV-positive.

3. Statistical analysis

P values less than 0.05 were considered statistically significant. The non-parametric Mann-
Whitney U-test was used to compare numerical variables between two groups, and binary
variables were analysed with contingency table analysis and Fisher’s exact test. The non-
parametric Spearman’s test was utilized in analyzing correlations between two numerical
variables. We calculated the overall survival from the date of MCC diagnosis until death by
the malignancy or the end of follow up. If a patient was alive at the end of follow-up, they
were censored from survival analysis. Survival was analysed with the Kaplan-Meier
method analysis, and the comparisons between two groups with log-rank test and Cox
regression analysis.
4. Atypically young MCC patients (Study I)

In study I, we combined data on clinical characteristics and tumour biology in an attempt to elucidate the characteristics of unusually young MCC patients. Patients 50 years or younger at the time of MCC diagnosis were sorted out from the histologically confirmed MCC patients diagnosed in Finland in 1979 to 2004. The clinical data listed above was compiled in detail for each patient. The stage of the disease at the time of diagnosis was defined according to the Memorial Sloan Kettering Cancer Center Staging system, which was the generally used system at the time (Table 5) (Allen et al. 2005). The follow-up for survival ended at 26th November 2006.

MCV analysis and survival data collection had been carried out for most of the patients diagnosed between 1979 and 2004 in our previous study (Sihto, et al. 2009). This data was used to compare the survival and MCV prevalence between the group of the younger MCC patients and the older patients.

<table>
<thead>
<tr>
<th></th>
<th>Primary tumour</th>
<th>Nodal involvement</th>
<th>Systemic metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>stage I</td>
<td>&lt; 2 cm</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>stage II</td>
<td>≥ 2cm</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>stage III</td>
<td>any T</td>
<td>≥ N1</td>
<td>-</td>
</tr>
<tr>
<td>stage IV</td>
<td>any T</td>
<td>≥ N1</td>
<td>≥ M1</td>
</tr>
</tbody>
</table>

Table 5: the Memorial Sloan Kettering Cancer Center Staging system for MCC. (Allen et al. 2005).
5. Bcl-2 expression in MCC (Study IV)

In study II, we evaluated the expression of the antiapoptotic protein bcl-2 by means of immunohistochemistry, and then analysed the possible trends arising in MCCs with differing courses of disease and etiological factors. The clinical data described above and MCV statuses were charted for the 116 patients. Special notice was allocated to the tumour location in order to assess the effects of UVR exposure to bcl-2 expression. The staging of the disease at the time of diagnosis was done according to AJCC staging system described on page 28. The end point of survival surveillance was 1st August 2008.

5.1. Immunohistochemistry

**Phase I:** Phase I was conducted to test the staining protocol and to find the most representative staining result groups for further analysis. We randomly selected 30 MCC patients diagnosed in 1979 to 2004 and stained one slide of their primary tumour block for bcl-2. Two researchers (Helka Sahi and Tom Böhling) interpreted the staining results and divided them with one accord into groups of negative and positive. The positive group was further divided into strongly and weakly positive categories.

**Phase II:** We selected tumour samples from 137 MCC patients diagnosed during 1979-2004 for phase II on the condition that there was sufficient high-quality material available for the multi-tissue array. A multi-tissue array made up of 417 cores from the 137 tumour samples was constructed.

The array was stained for bcl-2 with Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark) (IV). Only a few specimens were interpreted as weakly positive. These were included into the negative group, which was viewed as one in the succeeding statistical analysis. Inadequate specimens that were, for example, detached or folded over or did not contain tumour cells were censored from further analysis. At least one representative specimen was available for altogether 116 patients. The MCV status had been analysed from the tumours of 108 patients (Figure 7).
6. Chromosomal aberrations and RB1 hypermethylation (Study V)

We chose 15 MCV-negative and 15 positive MCC samples for the study on the conditions that MCV status had been evaluated, and there was enough paraffin-embedded tumour material available for DNA extraction. The samples were first analysed by the array genomic hybridization method (aCGH) to reveal possible chromosomal aberration distinctive for MCV tumours. Frequent heterozygous losses of the RB1 locus in MCV-negative tumours were detected. This led us to study the methylation pattern of the RB1 promoter region and to compare the findings to RB expression detected in our previous study by immunohistochemistry (Sihto et al. 2011).
6.1. Construction of aCGH microarrays and data analysis
QIAamp DNA mini Kit (Qiagen, Valencia, CA) was used to extract DNA from the paraffin-embedded tumour blocks. An adequate amount of DNA was extracted from 13 MCV-positive and 13 negative samples, out of which we also gathered two pools representing all MCV-positive and all MCV-negative samples. The Finnish Red Cross provided the reference DNA extracted from pooled male and female lymphocyte samples.

Digestion, labeling and hybridization of the samples was done according to Agilent’s Oligonucleotide Array-Based CGH for Genomic DNA analysis protocol version 6.1 (Agilent Technologies, Santa Clara, CA). Each labeled tumour DNA sample and the pooled DNA samples from MCV-positive and negative tumours were hybridized on 41 kb (60K) platforms. The pooled genome samples of MCV-negative tumours with MCV-positive tumour DNA samples as reference were further analysed with a higher 8.9 kb (244K) resolution platform.

We scanned the microarray slides with Agilent DNA microarray scanner (G2505C), and extracted the data with Agilent’s Feature Extraction (v.1.5.1.0.). The data was analysed with Agilent CytoGenomics software (v.2.0.6.0) Aberration Detection Method 2 algorithm (ADM2) after normalization. Gains were classified as high-level amplifications if log2 ratio was higher than 1 and losses as homozygous if log2 ratio was lower than 1. Annotations were derived from the Human Genome build 19, and the gene contents of minimal overlapping regions were re-checked from the gene database of the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/mapview/..

6.2. Detection of RB1 promoter methylation
Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) technique was employed to analyse the samples for possible hypermethylation of the RB1 promoter region. MS-MLPA probes bind to the normally unmethylated CpG-rich areas located near the promoter region of a given gene, and the amplification of those regions by endonucleases is dependent on the methylation of the region (Nygren et al. 2005).
DNA for MS-MLPA was extracted similarly to the DNA used in the aCGH analysis. We used a MS-MLPA probemix containing 5 probes for the RB1 promoter and 11 control probes, and MLPA reagents (EK1 kit; www.mlpa.com). MS-MLPA reactions were conducted twice according to the manufacturer’s instructions. PCR was also conducted accordingly (MRC-Holland). Coffalyser.NET software was used for the PCR fragment and data analysis (Coffa et al. 2011). References consisted of five samples of Promega reference DNA from pooled male and female blood samples, three paraffin-embedded normal skin samples and one paraffin block from a MCC patient lacking tumour cells.

6.3. RB immunohistochemistry
RB expression was studied by immunohistochemistry in our previous study for all but one of the samples (Sihto et al. 2011). A mouse monoclonal antibody (1F8; Thermo Fisher Scientific.; dilution 1:200) was used to detect the expression of RB from tissue microarrays. A breast cancer tissue microarray served as the positive control. The sample was considered RB immunopositive when the percentage of stained cells surpassed 20%.

EPIDEMIOLOGICAL STUDIES II AND III

The epidemiological studies II and III were carried out in close co-operation with the FCR, and are purely record-based. Reporting encountered cancer cases to the FCR is obligatory for all Finnish hospitals, physicians and pathological and haematological laboratories. Data on cancer cases is also gathered annually from death certificates with cancer registered as a cause of death. The FCR communicates regularly with the Population Register Center to keep up with deaths and emigration. Personal identity codes (PICs) have been in use in Finland since 1967, and they are utilized by all major registries to ensure safe and consistent record linkage that can be easily automated. The PIC was used as key in the record linkages in studies II and III.
Figure 8: The FCR is automatically linked to the Population Register Centre for data on death and immigration. For research purposes, information on drug reimbursements and purchases can be transported to the FCR for analysis. Personal identity codes (PICs) are used to ensure a secure and precise record linkage.

The National Social Insurance Institution (SII) manages a Prescription Register that gathers data on the reimbursements of expenses for medicines. Reimbursements that partly cover the expenses of registered drugs are admitted to all patients. In addition, patients with certain chronic diseases are entitled to complete reimbursements of drug expenses, providing a medical diagnosis with standardized terms. Purchases are registered with detailed information on the amounts of medicine and date of purchase. The database also provides information on the underlying chronic diseases warranting the upper categories of reimbursement, including the time of the diagnosis. The reimbursed drugs are classified according to the Anatomical Therapeutic Chemical (ATC) classification system, an international coding system that is generally accepted as the golden standard.
for drug-consumption studies. It is also supported by the World Health Organization (WHO).

1. Autoimmune diseases and MCC (Study II)
In study II, we aimed to elucidate the effect that ADs have on the risk of MCC. We used a retrospective case-control setting in which the incidence of ADs in MCC patients was compared with that in random control subjects. The cases were the patients with MCC diagnosed between 1964 and 2009 in Finland. 15 random persons per each MCC case matched for gender and year of birth were selected from the Population Register Centre as potential controls. The persons that were alive at the time of the MCC-diagnosis of the case were subsequently selected as the final controls.

Information on drug reimbursements was available up to 2010. The chronic diseases entitling patients to the upper categories of reimbursement are grouped by the SII into rather broad categories. Out of these categories, those that consisted mainly of chronic diseases accepted generally as ADs were chosen for further analysis (Table 6). We could not exclude the non-AD diagnoses from the broad categories, because the AD diagnoses and the respective ICD codes had not been registered before the year 2000. The SII categories were subsequently divided into site-specific groups seen in Table 6.

ADs were listed until the date of the MCC diagnosis for both cases and controls. We used a conditional regression model and the odds ratio (OR) to estimate the risk of MCC associated with ADs. 95% confidence intervals were calculated. Time-dependent factors were estimated with the date of the first diagnosed AD.

2. Statins and MCC (Study III)

In study III, we examined Finnish statin users in a cohort study in order to find out the influence of statin use on the incidence of MCC. A cohort of patients with registered purchases of statins during 1994-2007 was identified from the Prescription Register.
<table>
<thead>
<tr>
<th>SII-group</th>
<th>AD</th>
<th>ICD-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connective tissue/Systemic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>202 Rheumatoid conditions</td>
<td>Langerhans cell histiocytosis not elsewhere classified</td>
<td>D76.0</td>
</tr>
<tr>
<td></td>
<td>Other histiocytosis syndromes</td>
<td>D76.3</td>
</tr>
<tr>
<td></td>
<td>Chronic iridocyclitis</td>
<td>H20.1</td>
</tr>
<tr>
<td></td>
<td>Crohn's disease, unspecified</td>
<td>K50.9</td>
</tr>
<tr>
<td></td>
<td>Ulcerative colitis, unspecified</td>
<td>K51.9</td>
</tr>
<tr>
<td></td>
<td>Autoimmune hepatitis</td>
<td>K75.4</td>
</tr>
<tr>
<td></td>
<td>Primary biliary cirrhosis</td>
<td>K74.3</td>
</tr>
<tr>
<td></td>
<td>Ankylosing spondylitis</td>
<td>M45</td>
</tr>
<tr>
<td></td>
<td>Arthropathic psoriasis</td>
<td>L40.5</td>
</tr>
<tr>
<td></td>
<td>Postinfective and reactive arthropathies</td>
<td>M02</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid arthritis with rheumatoid factor</td>
<td>M05</td>
</tr>
<tr>
<td></td>
<td>Other rheumatoid arthritis</td>
<td>M06</td>
</tr>
<tr>
<td></td>
<td>Juvenile arthritis</td>
<td>M08</td>
</tr>
<tr>
<td></td>
<td>Relapsing polychondritis</td>
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</tr>
<tr>
<td></td>
<td>Polyartentis nodosa and related conditions</td>
<td>M30</td>
</tr>
<tr>
<td></td>
<td>Other necroting vasculopathies</td>
<td>M31</td>
</tr>
<tr>
<td></td>
<td>Systemic lupus erythematosus (SLE)</td>
<td>M32</td>
</tr>
<tr>
<td></td>
<td>Dermatopolymyositis</td>
<td>M33</td>
</tr>
<tr>
<td></td>
<td>Systemic sclerosis</td>
<td>M34</td>
</tr>
<tr>
<td></td>
<td>Other systemic involvement of connective tissue</td>
<td>M35</td>
</tr>
<tr>
<td></td>
<td>Unspecified inflammatory spondylopathy</td>
<td>N03</td>
</tr>
<tr>
<td></td>
<td>Arthritis, unspecified</td>
<td>M13.9</td>
</tr>
<tr>
<td></td>
<td>Sacroilitis, not elsewhere classified</td>
<td>M46.1</td>
</tr>
<tr>
<td></td>
<td>Chronic nephritic syndrome</td>
<td>M46.9</td>
</tr>
<tr>
<td></td>
<td>Cholangitis</td>
<td>Q44.2</td>
</tr>
<tr>
<td></td>
<td>Atresia of bile ducts</td>
<td>K83.0</td>
</tr>
<tr>
<td></td>
<td>Enterosis due to Yersinia enterocollitica</td>
<td>A04.6</td>
</tr>
<tr>
<td></td>
<td>Other meningococcal infections</td>
<td>A39.8</td>
</tr>
<tr>
<td></td>
<td>Other late congenital syphils, symptomatic</td>
<td>A50.5</td>
</tr>
<tr>
<td></td>
<td>Chorioretinal inflammation</td>
<td>H30</td>
</tr>
<tr>
<td></td>
<td>Acute and subacute infective endocarditis</td>
<td>I33.0</td>
</tr>
<tr>
<td></td>
<td>Other interstitial pulmonary diseases</td>
<td>J84</td>
</tr>
<tr>
<td></td>
<td>Chronic active hepatitis, not elsewhere classified</td>
<td>K73.2</td>
</tr>
<tr>
<td>132 Sarcoïdosis</td>
<td>Sarcoidosis</td>
<td>D86</td>
</tr>
<tr>
<td></td>
<td>Acquired pure red cell aplasia</td>
<td>D60</td>
</tr>
<tr>
<td>122 Ablastic anaemia</td>
<td>Other aplastic anemias and other bone marrow failure syndrome</td>
<td>D61</td>
</tr>
<tr>
<td>Gi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>208 Colitis ulcerosa, Morbus Crohn</td>
<td>Morbus Chron</td>
<td>K50</td>
</tr>
<tr>
<td></td>
<td>Colitis ulcerosa</td>
<td>K51</td>
</tr>
<tr>
<td>Skin</td>
<td></td>
<td></td>
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<tr>
<td>135 Pemphigus</td>
<td>Pemphigus</td>
<td>L10</td>
</tr>
<tr>
<td></td>
<td>Pemphigoid</td>
<td>L12</td>
</tr>
<tr>
<td></td>
<td>Lichen planus</td>
<td>L43</td>
</tr>
<tr>
<td></td>
<td>Psoriasis</td>
<td>L40</td>
</tr>
<tr>
<td></td>
<td>Systemic lupus erythematosus</td>
<td>M32</td>
</tr>
<tr>
<td>134 General erythrodermia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>110 Parkinson's disease, comparable disorders</td>
<td>Parkinson's disease</td>
<td>G20</td>
</tr>
<tr>
<td>109 Multiple sclerosis</td>
<td>Multiple sclerosis</td>
<td>G35</td>
</tr>
<tr>
<td>108 Myasthenia gravis</td>
<td>Myasthenia gravis</td>
<td>G70.0</td>
</tr>
<tr>
<td>Neural</td>
<td></td>
<td></td>
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<td>103 Diabetes mellitus</td>
<td>Type 1 diabetes mellitus</td>
<td>E10</td>
</tr>
<tr>
<td></td>
<td>Type 2 diabetes mellitus</td>
<td>E11</td>
</tr>
<tr>
<td></td>
<td>Unspecified diabetes</td>
<td>E14</td>
</tr>
<tr>
<td></td>
<td>Other specified diabetes mellitus</td>
<td>E13</td>
</tr>
<tr>
<td></td>
<td>Diabetes due to malnutrition</td>
<td>E12</td>
</tr>
<tr>
<td></td>
<td>Postprocedural hypoinsulinemia</td>
<td>E89.1</td>
</tr>
</tbody>
</table>

**Table 6**: Social Insurance Institution (SII) groups and ADs included in the study. Disease entities that are imprecise or include non-AD are marked with italics.
The statins and their ATC codes are listed in Table 7. The beginning of MCC follow-up was the date of the first statin purchase, and the end of follow-up was either the date of death or 31st December 2009. Follow-up time was divided into categories of 0, 1 to 4 years, and 5 or more years, based on the duration of statin use in complete years calculated from the date of the first registered purchase.

<table>
<thead>
<tr>
<th>Statin</th>
<th>ATC code</th>
</tr>
</thead>
<tbody>
<tr>
<td>simvastatin</td>
<td>C10AA01</td>
</tr>
<tr>
<td>lovastatin</td>
<td>C44M8247</td>
</tr>
<tr>
<td>pravastatin</td>
<td>C10AA03</td>
</tr>
<tr>
<td>fluvastatin</td>
<td>C10AA04</td>
</tr>
<tr>
<td>atorvastatin</td>
<td>C10AA05</td>
</tr>
<tr>
<td>serivastatin</td>
<td>C10AA06</td>
</tr>
<tr>
<td>rosuvastatin</td>
<td>C10AA07</td>
</tr>
</tbody>
</table>

*Table 7*: The statins included in the study with their ATC codes.

Person-years at risk and observed cases were counted separately for groups determined by sex, four year calendar periods beginning from 1994, and five year age groups. The calculation of the expected numbers of cases and the standardized incidence ratios is depicted in Figure 9. 95% CIs for SIR were calculated assuming that the number of observed cases followed a Poisson distribution. We used the Poisson regression model to analyse trends of MCC incidence according to the groups listed above. We also analysed statin groups separately so that the first statin purchased determined the statin group of the patient.

\[
\text{SIR} = \frac{\text{Observed cases}}{\text{Expected cases}} = \frac{\text{Person-years} \times \text{MCC incidence in comparable population}}{\text{Expected cases}}
\]

*Figure 9*: Standardized incidence ratios (SIRs) were calculated by dividing the number of observed cases of MCC with the expected cases of MCC. The numbers of expected cases were estimated by multiplying the person-years of the statin users in each group with the reported incidence of MCC in gender and age-adjusted Finnish population.
RESULTS

UNUSUALLY YOUNG MCC PATIENTS (STUDY I)

181 patients had been diagnosed with MCC in Finland during 1979-2004. Six of these patients (3.31%) were 50 years or younger at time of diagnosis (range 27-50 years). Five of the patients had a history of some sort of an immunodeficiency, three of which were ADs. MCV status could not be analysed for one of the patients, as she presented with nodal metastasis without a primary tumour. All of the analysed tumours contained MCV DNA. The immunocompromising states, clinical characteristics of the patients, MCV status are listed in Table 8.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Immunodeficiency</th>
<th>MCV copy number</th>
<th>Follow-up (years)</th>
<th>Alive at the end of follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>F/27</td>
<td>Pregnancy</td>
<td>6.942</td>
<td>2.1</td>
<td>Yes</td>
</tr>
<tr>
<td>2.</td>
<td>M/35</td>
<td>None</td>
<td>1.18545</td>
<td>17.0</td>
<td>Yes</td>
</tr>
<tr>
<td>3.</td>
<td>M/47</td>
<td>Sarcoidosis, DMII</td>
<td>1.568</td>
<td>16.7</td>
<td>Yes</td>
</tr>
<tr>
<td>4.*</td>
<td>F/50</td>
<td>Psoriasis; topical cortisone treatment</td>
<td>-</td>
<td>5.1</td>
<td>Yes</td>
</tr>
<tr>
<td>5.</td>
<td>F/50</td>
<td>Mycosis fungoides, Psoriasis; PUVA, RT and interferon treatment</td>
<td>0.00053</td>
<td>1.1</td>
<td>No</td>
</tr>
<tr>
<td>6.</td>
<td>M/44</td>
<td>RA, renal transplant; azathioprine and cortisone treatment</td>
<td>5.448</td>
<td>2.3</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 8: Clinical and tumour characteristics of unusually young MCC patients diagnosed in 1979-2004. Rheumatoid arthritis (RA), diabetes mellitus II (DMII), psoralen and UVA therapy (PUVA), radiation therapy (RT).

*No detectable primary tumour.
The mean follow-up time was 10 years (range 2-17 years). A slight trend towards a worse MCC-specific survival was noted in Kaplan Meyer analysis: the five-year survival of younger MCC patients was 62.5% and 72.3% for patients older than 50 years. The differences in MCC-specific or overall survival did not reach statistical significance.

**AUTOIMMUNE DISEASES AND MCC (STUDY II)**

270 patients had been diagnosed with MCC during 1964 to 2009. The number of cases included in this study decreased to 267 due to a lack of matching controls for three of the MCC patients. There were 3270 controls.

<table>
<thead>
<tr>
<th>ADs by organ system*</th>
<th>N MCC (%)</th>
<th>N Controls (%)</th>
<th>OR</th>
<th>95 % CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systemic/connective tissue AD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rheumatoid conditions</td>
<td>22 (8.2%)</td>
<td>140 (4.3%)</td>
<td>1.96</td>
<td>1.22-3.15</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ablastic anaemia</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dermatologic AD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrodermia</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pemphigus</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gastrointestinal AD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8</td>
<td>1.59</td>
<td>0.20-12.8</td>
</tr>
<tr>
<td><strong>Neural AD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parkinson's disease</td>
<td>5</td>
<td>72</td>
<td>0.78</td>
<td>0.30-2.00</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>0</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Diabetic conditions</strong></td>
<td>35 (13.1%)</td>
<td>296 (9.1%)</td>
<td>1.51</td>
<td>1.03-2.22</td>
</tr>
</tbody>
</table>

*Table 9: The numbers (N) of autoimmune diseases (ADs) in cases with Merkel cell carcinoma (MCC) and controls divided into site-specific groups. Odds ratios (ORs) are listed with their 95% confidence intervals (95%CI). *List of ADs is found in Table 6.*
 Altogether, 59 (22.1%) of the MCC patients had been diagnosed with at least one AD, whereas the respective number for the controls was 487 (14.9%). Diagnosis of any AD or several lead to a significantly increased risk for MCC (OR 1.63, 95%CI 1.19-2.22). The time between AD and MCC diagnosis ranged from 1-40 years (mean 14.3 years, standard deviation 9.3 years). The OR was not dependent on time between the diagnoses (≤10 years, OR 1.70, 95% CI 1.04-2.79; >10 years, OR 1.77, 95%CI 1.22-2.57). The gender or the ages of the MCC patients were not significantly associated with the occurrence of AD.

History of ADs of the ‘connective tissue/systemic’ group increased the risk for MCC significantly (OR 2.01, 95% CI 1.26-3.20, Table 9). The increased risk was mainly due to the effect of rheumatoid conditions, which themselves had an estimated OR of 1.96 (95% CI 1.22-3.15). In addition, diabetic conditions significantly increased the risk for MCC (OR 1.51, 95% CI 1.03-2.22). Neural and gastrointestinal ADs were sparse and did not increase the risk for MCC significantly.

STATINS AND MCC (STUDY III)

The cohort of statin users consisted of 454 937 persons: 224 716 (49%) men and 230 221 (51%) women. 56% of the patients were over 60 years at time of first statin purchase. The mean length of follow-up was 9.2 years. The numbers of statin users and person-years at risk are described in more detail in Table 10.

50 MCC cases were diagnosed among the study cohort during the follow-up. The expected number of MCCs based on the comparable, age-adjusted, Finnish population was 39.9 (SIR 1.25, 95%CI 0.93-1.65). Age-specific SIRs and observed cases are listed in Table 11. Every 5 year ascent in age decreased the relative risk of MCC 0.79 fold (95% CI 0.67-0.92). No significant differences in SIR related to the length of follow-up or gender were observed. None of the statins lead to significant findings when examined singly.
<table>
<thead>
<tr>
<th>Men N</th>
<th>Women N</th>
<th>All N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Person-years</td>
<td>Person-years</td>
<td>Person-years</td>
</tr>
<tr>
<td>&lt;30 years</td>
<td>1 456</td>
<td>1 303</td>
</tr>
<tr>
<td>7 024</td>
<td>7 828</td>
<td>14 852</td>
</tr>
<tr>
<td>30-59 years</td>
<td>118 680</td>
<td>79 200</td>
</tr>
<tr>
<td>805 106</td>
<td>454 704</td>
<td>1 259 810</td>
</tr>
<tr>
<td>≥ 60 years</td>
<td>104 580</td>
<td>149 718</td>
</tr>
<tr>
<td>1 240 978</td>
<td>1 663 736</td>
<td>2 904 714</td>
</tr>
<tr>
<td>TOTAL</td>
<td>224 716</td>
<td>230 221</td>
</tr>
<tr>
<td>2 053 109</td>
<td>2 126 269</td>
<td>4 179 378</td>
</tr>
</tbody>
</table>

Table 10: Numbers (N) of statin users and person-years in the study cohort, by sex and age. Age was defined at the beginning of follow-up.

<table>
<thead>
<tr>
<th>Age</th>
<th>Obs</th>
<th>Exp</th>
<th>SIR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;60 years</td>
<td>3</td>
<td>0.95</td>
<td>3.16</td>
<td>0.65-9.23</td>
</tr>
<tr>
<td>60-74 years</td>
<td>23</td>
<td>11.88</td>
<td>1.94</td>
<td>1.23-2.90 *</td>
</tr>
<tr>
<td>75+ years</td>
<td>24</td>
<td>27.08</td>
<td>0.89</td>
<td>0.57-1.31</td>
</tr>
<tr>
<td>All</td>
<td>50</td>
<td>39.9</td>
<td>1.25</td>
<td>0.93-1.65</td>
</tr>
</tbody>
</table>

* p < 0.01

Table 11: Observed (Obs) and expected (Exp) numbers of MCC cases in 1994-2007, by 15 year age-groups. Standardized incidence ratios (SIR) are listed with their 95% confidence intervals (95% CI).

BCL-2 EXPRESSION IN MCC (STUDY IV)

18 (60.0%) of the 30 tumours included in phase I were strongly positive for bcl-2, 5 (16.7%) were weakly positive and 7 (23.3%) negative (Figure 10). If bcl-2 positivity was observed, all of the tumour cells were stained (Figure 10). 99 of the 116 tumour samples (85.3%)
represented in the multi-tissue array were bcl-2 positive and 17 (14.7%) negative. The negative group contained the weakly positive samples as described earlier. Bcl-2 expression and the presence of MCV DNA were not significantly associated: 80.4% of the bcl-2 positive samples and 75.0% of the bcl-2 negative samples were MCV-positive (p=0.737). The groups did not differ significantly in gender spectrum, age or the distribution of tumour location.

Figure 10: MCC tumours showing bcl-2 negative (A), weakly positive (B) and strongly positive (C) immunostaining. In the bcl-2 positive MCCs, all tumour cells are stained. Original magnification x 200.

There was a trend towards smaller tumours in the bcl-2 positive group, but the difference was not statistically significant (p=0.114). The local recurrence rates did not vary significantly between the two groups, but patients with bcl-2 positive tumours were less likely to have nodally or systemically metastatic MCCs (33.3% vs. 11.9%, p=0.044). The follow-up time ranged from 5 days to over 22 years (mean 4.6 years). 22.2% of the patients with bcl-2 positive tumours survived until the end of follow up, as opposed to none.
of the patients with bcl-2 negative tumours (p=0.039). The mean overall survival was 5.0 years in patients with bcl-2 positive tumours and 2.1 years in patients with bcl-2 negative MCC (p=0.010). The differences in overall survival observed with Kaplan-Meier analysis (Figure 11) were statistically significant (p=0.0017).

![Figure 11](image)

**Figure 11:** Overall cumulative survival in years analysed with the Kaplan-Meier method. The percentage of patients alive is marked on the vertical axis, and the survival in days on the horizontal axis. Patients with bcl-2 positive MCC are marked with a blue line and patients with bcl-2 negative MCC with a red line. The difference in overall survival was statistically significant (p=0.0017).

CHROMOSOMAL ABERRATIONS AND RB1 HYPERMETHYLATION (STUDY V)

1. Array CGH results and clinical data

DNA could be extracted in sufficient amounts from 13 MCV-positive tumours and 13 MCV-negative tumours. The most frequent changes included the losses of chromosomes 3p, 4 and 11p, as well as the gains of chromosomes 11, 12 and 14. Diagrams showing the copy
Figure 12: Graphic compilations of the aberrations detected by aCGH. Pooled MCV-negative (a) and MCV-positive (b) samples against male reference with 60k resolution. Pooled MCV-negative samples against pooled MCV-positive samples as reference, with 60k (c) and 244 k (d) resolution. Figures were prepared with Agilent CytoGenomics software (v.2.0.6.0).
number changes in pooled virus positive versus negative samples samples are shown in Figure 12. The numbers of chromosomal aberrations were not associated with the stage of the disease or survival. Narrow region gains and losses were frequent. The most common minimal overlapping regions are listed in Table 12.

<table>
<thead>
<tr>
<th>Location</th>
<th>Size (kb)</th>
<th>Number of samples</th>
<th>Number of genes</th>
<th>Genes of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplifications</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p34.2</td>
<td>3 570</td>
<td>3</td>
<td>74</td>
<td>MYCL1, RLF, MYCBP, RRAGC, CDCA8</td>
</tr>
<tr>
<td>6p22.3</td>
<td>260</td>
<td>3</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>11q13.4</td>
<td>103</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Gains</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1p35.2</td>
<td>1 830</td>
<td>3</td>
<td>27</td>
<td>PBXIP1, RIT1, RAB25, VHL, PRCC</td>
</tr>
<tr>
<td>1q21.3</td>
<td>2 620</td>
<td>6</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>1q32.1</td>
<td>1 850</td>
<td>5</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>3q26.3</td>
<td>850</td>
<td>4</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>6p22.3</td>
<td>270</td>
<td>7</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Losses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5q23.1</td>
<td>850</td>
<td>6</td>
<td>19</td>
<td>CSF2, RAC1P3, RGCC, TNFSF11, EPSTI1, TPT1, RB1</td>
</tr>
<tr>
<td>13q14.11</td>
<td>9 190</td>
<td>6</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>17p11.2</td>
<td>380</td>
<td>7</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

Table 12. Common narrow region copy number changes detected in the 26 MCC tumour samples. The number of all genes located in the minimal overlapping regions genes is listed along with genes of interest.

MCV-negative tumours were distinctly smaller than MCV-positive tumours with the mean diameter of 19.9 mm compared to 37 mm (p=0.031). Viral status did not correlate significantly with survival. The aberrations detected in MCV-negative and positive tumours are shown as diagrams in Figure 1. MCV-negative presented with significantly more frequent copy number losses than MCV-positive tumours (mean 6.6. per tumour vs. 2.3 per tumour, p=0.0150) respectively. (Figure 12)
Gains of chromosomes 5p, 11 and 12 were common in MCV-positive tumours, whereas MCV-negative tumours were characterized by the amplification of the narrow region 1p34.3-1p34.2, and the losses of chromosomes 3p, 11p, and 13. Three MCV-negative samples presented with 1p34.3-1p34.2 amplification, which contains the proto-oncogene L-Myc. No amplifications of 1p34.3-1p34.2 were detected in MCV-positive tumours (p=0.220). Six samples presented with a deletion of 13q14.1-13q21.3, which contains the RB1 locus, and four of them were MCV-negative (Table 3, p=0.160). Deletions of the RB1 locus did not have a significant effect on survival. Only the deletions of chromosome 11p seen more often in MCV-negative tumours were statistically significant when comparing the differences between the two groups (p=0.039).

2. RB expression

RB immunohistochemistry was available for all but one sample. RB was expressed in 10/13 MCV-positive tumours and 2/13 MCV-negative tumours (p=0.005). Samples with RB1 deletions did not express RB. RB expression was associated with a statistically significantly better survival detected by Kaplan Meyer analysis (p=0.027, Figure 13).

Figure 13: The vertical axis depicts the percentage of patients alive, and the horizontal axis survival in days. Patients whose MCC tumours expressed RB (blue line) showed a trend for better overall survival compared to those whose did not (red line, p=0.154).
3. Methylation of the RB1 promoter

Adequate DNA could not be extracted from one of the tumour samples. The remaining 25 tumour samples presented with a low methylation signal (<26%) of the RB1 promoter with two consequent probes of the five probes included in the mixture (Table 13). Single low-methylation signals were also detected with the remaining three probes. The pooled blood control samples were not methylated in any part of the RB1 promoter region, whereas the normal skin samples presented with low methylation signals ranging from 5% to 7% detected with a single probe.

No associations were detected between the methylation of any of the probe-spanned promoter regions and survival, RB expression, deletions of RB1, or MCV status. We also compared the tumours methylated only in the areas of two probes showing consistent methylation to those with methylation also in the areas of any of the remaining three probes. No significant differences were detected in survival, RB expression, RB1 deletion or viral status.

<table>
<thead>
<tr>
<th>Probe</th>
<th>MCC samples mean (range)</th>
<th>Normal skin 1</th>
<th>Normal skin 2</th>
<th>Normal skin 3</th>
<th>Promega female blood references x 2</th>
<th>Promega male blood references x3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 (0-4%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2% (0-6%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0 (0-11%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>14% (8-23%)</td>
<td>5%</td>
<td>6%</td>
<td>7%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>13% (7-26%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 13. The methylation percentages of the RB1 promoter areas detected with each probe. Probe no.1 spanned the area furthest away from ATG start codon and probe no. 5 respectively the nearest.
DISCUSSION

UNUSUALLY YOUNG MCC PATIENTS (STUDY I)

Merkel cell carcinoma is first and foremost a disease of the elderly, which makes it even more intriguing to try and fill the gap of pathologic and epidemiologic knowledge that the few young patients present. To the best of our knowledge, ours was the first study conducted to shed light on the MCC of patients 50 years or younger. Previously, only case reports had depicted MCC in atypically young patients.

The proportion of MCCs presenting in patients 50 years or younger has been calculated as approximately 3% (Kukko et al. 2012; Kaae et al. 2010). In our material, the proportion was 3.5%. Only single MCC cases appear in patients before the age of 55-60 years, after which the incidence rises abruptly, being approximately 1/100 000 at 75 and 2/100 000 at 85 (Kukko et al. 2012; Kaae et al. 2010; Reichgelt et al. 2011). According to a recent Danish study, the rise in MCC incidence seen in the past decades most obviously affects patients older than 65 years (Lyhne et al. 2011). In this respect, immunosenescence and increased UV exposure could be thought as being the main culprits behind the recent rises of MCC incidence.

Five of the six unusually young MCC patients suffered from a reported immunodeficiency. Surprisingly, four of the patients had a history of AD. All of these patients had received immunosuppressive medication in the treatment of their condition. Furthermore, overlapping immunosuppressive conditions were found in three of the AD patients: DMII in the patient with sarcoidosis; mycosis fungoides with PUVA interferon treatment in on the psoriasis patient; and renal transplancy due to amyloidosis in the RA patient. A noteworthy point was the absence of HIV and AIDS, which have been depicted as predispositional factors for MCC at a young age in various case reports (Manganoni et al. 2007; Matichard
et al. 2002; Samarendra et al. 2000). The lack of HIV and AIDS among our patients is best explained by the low incidence of HIV infection in the Finnish population in global comparison and aggressive treatment protocols.

The portion of immunocompromised patients among all MCC cases is approximately 6-8.7%, compared to the 85% among our young MCC patients (Girschik et al. 2011; Paulson et al. 2013; Heath et al. 2008). This finding is in line with the reports that MCC patients with a history of immunosuppression are diagnosed at a distinctly earlier age than the average (approximately 60 years vs. 75 years) (Penn et al. 1999; Koljonen et al. 2009b; Engels et al. 2002). There was also a trend towards a worse disease-specific prognosis in the unusually young MCC patients, which is in accordance with reports depicting the more aggressive nature of MCC in immunocompromised patients (Penn et al. 1999; Brewer et al. 2012; Paulson et al. 2013; Koljonen et al. 2009b).

All of the available primary tumours of our unusually young MCC patients harboured MCV DNA. Only a few studies have addressed the age distribution of MCV infected MCC patients. Bhatia and co-workers analysed the MCV status of 23 MCCs, and reported that patients with MCV-positive tumours were significantly younger than those with MCV-negative tumours. No differences in the age-spectrum were revealed in our previous large-scale study that compared the clinical features of 114 MCV-positive and negative MCCs. MCV copy numbers varied widely from 0.00053 to 6.9 per reference gene among the younger MCC patients. During the time of the study, we interpreted all tumours with any MCV DNA content as ‘MCV-positive’. Later on, it was established that only a part of these tumours expressed LT (Sihto et al. 2011). The cut-off copy number value for MCV-positive has since been set to 0.1 per reference gene, and according to modern standards, the MCC tumour of patient n:o 5 (Table 8) would now be interpreted as MCV-negative.

**AUTOIMMUNE DISEASES AND MCC (STUDY II)**

In study II, we found that MCC patients had more often been diagnosed with ADs than the average age and sex matched Finnish population. MCC and ADs with ‘connective
tissue/systemic' involvement were strongly connected. The vast majority of AD diagnoses fell in to the group of rheumatoid conditions.

Only two studies have been conducted to assess the role of ADs as predictors of MCC separately from other NMSC. Hemminki and co-workers gathered a cohort of AD patients from the Swedish Hospital discharge register and compared the MCC incidences to the standard population to produce SIRs (Hemminki et al. 2012). They found that the risk for MCC was increased among patients with inflammatory bowel disease and ankylosing spondylitis. Lanoy and Engels conducted a SEER-based case-control study focusing on elderly skin cancer patients (≥67 years), including MCC cases (Lanoy et al. 2011). A connection between RA and MCC was found. In our study, both RA and ankylosing spondylitis were included into the group of rheumatoid conditions, which was linked to an increased risk for MCC.

The pathogenesis of RA is a complex interplay of disturbances of cytokine responses, NK cell activity and the Th1/Th2-cell spectrum (Cope 2008; Elewaut 2005; Chen et al. 1993), all of which could hinder defence against MCV. Furthermore, rheumatoid conditions can lead to the reactivation of polyomaviruses BK and JC from a latent subclinical state to a lytic infection (Sundsfjord et al. 1999; Palazzo et al. 2012). In RA patients, the reactivation of the polyomavirus JC and subsequent progressive multifocal leukoencephalopathy is a well-known phenomenon (Palazzo et al. 2012; Marzocchetti et al. 2008). AD diagnoses preceded MCC diagnosis with 14.3 years on average, theoretically leaving adequate time for viral reactivation and the ensuing oncogenesis.

Diabetic conditions in general increased the risk for MCC (OR 1.51, 95% CI 1.03-2.22), although we could not examine diabetes mellitus I (DMI) separately from the non-autoimmune diabetic conditions. The incidences of gastrointestinal cancers, squamous cell carcinoma and leukaemia increase with DMI (Shu et al. 2010). Hyperglycemia in itself has been observed to promote autoimmunity through the regulation of the apoptosis of Tregs (Ramakrishnan et al. 2011). Diabetes mellitus II (DMII) is not an AD, but it is linked to an
increased risk of bowel, liver and pancreatic cancers (Tsugane et al. 2010). Added to this equation are the immunomodulatory and dermatological side-effects that glucose-lowering drugs, especially insulin, can exert (Van Hattem et al. 2008; Janghorbani et al. 2012).

The high coverage of FCR ensured technical completeness for MCC cases, and the lengthy time between AD and MCC diagnoses helped to avoid a surveillance bias. The coverage of the Prescription Register data is quite high in the cases of severe AD needing constant medication, as the patients are more likely to seek compensation. As for less severe diseases that only require intermittent or topical treatment, for example in the cases of psoriasis or inflammatory bowel diseases treated with 5-ASA, the coverage is likely less complete. The study protocol encountered its most pressing limitations due to the broad SII categories and unavailable ICD-coding. We could not include all AD, and some of the SII categories included non-autoimmune diseases. The diseases that are not considered to be of autoimmune origin are marked in italics in Table 6. These entities consisted mostly of chronic infectious and metabolic diseases characterized by chronic inflammation, and fortunately resemble AD in many of their immune responses.

Estimating the possible increases in cancer risk that autoimmune processes themselves account for is highly difficult in epidemiological settings, because of the immunomodulating drugs used in the management of the diseases. Furthermore, the severity of the AD often correlates to the intensity of the treatment. In study II, we would have had the possibility to use information on drug purchases starting from 1994. Unfortunately, we could not come up with a reasonable way of utilizing the lists of single purchases in determining the daily dosages of systemic drugs. Issues such as drug adherence, use of local treatment and drugs administered in hospitals confound the estimates.

The advent of biological disease-modifying antirheumatic drugs is such a recent event, that study II does not provide information on their effect on MCC risk. Biological antirheumatic drugs have already been reported to have lead to an increased risk of skin cancer in multiple sclerosis patients, even though they have normally have a lower risk of cancer in
general (Lebrun et al. 2011; Sumelahti et al. 2004). In our material, there were too few multiple sclerosis cases to draw any conclusions. This study protocol could be used to estimate the effect of biological antirheumatic drugs on cancer risk quite reliably in the future considering the precise recording and undoubtedly also good adherence to these valuable therapies.

STATINS AND MCC (STUDY III)

Study III was the first study ever to evaluate the effects of statin use on the incidence of MCC separately from other NMSC. It is also the first study that links a neuroendocrine carcinoma to statin use. We found that MCC is more common among statin users than the standard Finnish population before the age of 70. The SIRs decreased significantly when moving to older age groups. This finding is plausible considering the immunomodulatory effects of statins and the younger age distribution of immunosuppressed MCC patients (Penn et al. 1999; Koljonen et al. 2009b; Engels et al. 2002), but are contradictory to studies reporting increased risk of NMSC especially in the elderly (Shepherd et al. 2002; Mascitelli et al. 2012).

Lovastatin (Mevacor®) received a license from the FDA in 1987, and was first released in the Finnish market in 1988 (MSD Finland, personal communication). The battle to launch the HMG-reductase inhibitors in clinical use was slowed down in the last stages by the suspected increase in lymphomas in dogs treated with large doses of the statin compactin (Endo 2010). The effects of statins on cancer risk are researched rigorously, with meta-analyses reporting mostly neutral effects on the overall risk of cancer (Bonovas et al. 2006; Browning et al. 2007; Dale et al. 2006). A large Finnish record linkage study recently reported a slight trend suggesting a connection between pravastatin and non-melanoma skin cancers (Haukka et al. 2010). Similarly, a comprehensive meta-analysis by Kuoppala and co-workers suggested that the use of simvastatin would increase the cancer risk (Kuoppala et al. 2008). Our findings suggest that the use of statins does lead to an increased incidence of MCC, although no trend connecting longer follow-up time since the
first purchase to a higher increase in the MCC incidence was observed. This would have served as an indicator of dose-response, since statins are mostly used indefinitely after the diagnosis of hypercholesterolemia or vascular disease.

Many of the immunomodulatory effects of statins, including the pro-apoptotic effect leading to auto-antibodies and the disturbances in Th1/Th2 spectrum, characterize the pathogenesis autoimmunity (Rovere et al. 2000; Cope 2008). Some statins have also been reported to affect the peripheral pool of CD4+CD25+ Tregs (Mausner-Fainberg et al. 2008). ADs such as dermatomyositis, polymyositis, and SLE have been linked to statin use (Noel 2007). Paradoxically, the shift to the Th2 direction may actually be beneficial in the treatment of autoimmune diseases characterized by Th1-response, for example RA and psoriasis (McCarey et al. 2004; Rajpara et al. 2010; Egesi et al. 2010). In light of the discoveries of studies II and III, it could be thought that statins and ADs might have quite similar effects in the tumorigenesis of MCC.

The setting of study III has certain limitations. Patients with chronic vascular diseases are under close medical surveillance, which might lead to surveillance bias. In addition, some ADs are associated with high cholesterol levels, and the patients are more likely to use statins and start the medication at an earlier age (Rajpara et al. 2010; Lee et al. 2010). The record linkage setting is not optimal in estimating the exposure to statins, since adherence to drug use cannot be verified based solely on the registered purchases.

**BCL-2 EXPRESSION IN MCC (STUDY IV)**

In study IV, we detected frequent expression of the anti-apoptotic protein bcl-2 in MCC tumours by immunohistochemistry. Earlier smaller scale studies led by Feinmesser and Kennedy were unable to show significant correlations between bcl-2 expression and survival. Surprisingly, in our material, the patients with bcl-2 positive tumours were significantly less likely to present with metastatic MCC at the time of diagnosis. They were
also more likely to survive until the end of follow up, and their overall survival in days was longer compared to patients with bcl-2 negative tumours. No correlation between tumour size and bcl-2 expression were noted, even though tumour size remains the most reliable prognostic factor in MCC. Bcl-2 expression predicted better survival similarly in both MCV-negative and positive MCCs.

The evaluation of bcl-2 expression has been complex and variable in previous immunohistochemical studies (Kennedy et al. 1996; Feinmesser et al. 1999). The phase I of our study revealed that when a MCC tumour was positive for bcl-2, all of the tumour cells were stained. The intensity of the staining varied so that a small portion of the tumours were only weakly positive, which we included into the negative group. We recommend that in future studies MCC tumours be simply classified into groups of clearly bcl-2 positive and weakly positive/negative.

How is it that the expression of an anti-apoptotic protein could paradoxically lead to a better prognosis of a malignancy? The apoptotic effect is pursued with chemo- and radiation therapy, both of which have some effect in curbing MCC progression. The effects of the main regulators of bcl-2 functions, including phosphorylation and suppression by bax and c-kit, are difficult to foretell, and might partly explain our contradictory finding (Oltvai et al. 1993; Reed 1994; Moll et al. 1996a; Su et al. 2002). These complex interactions may even underlie the poor results reported regarding the in vivo testing of both bcl-2 antisense oligonucleotides and Kit-ligand tyrosine kinase inhibitors (Shah et al. 2009; Samlowski et al. 2010; Fenig et al. 2004), even though preliminary in vitro studies showed promising results (Schlagbauer-Wadl et al. 2000; Krasagakis et al. 2011).

The truncation of the LT follows MCV integration to the host cell genome, and has been shown to be particular to MCC tumours. (Shuda, et al. 2008) Truncation of LT renders the virus unable to replicate (Shuda, et al. 2008), which has been speculated to be vital for tumour progression. Ongoing replication would eventually lead to host cell lysis or such major genomic changes that apoptosis would inevitably ensue. (Houben, et al. 2009;
Olavarrieta, et al. 2002) In theory, the over expression of anti-apoptotic proteins could allow for genomic imbalances to go unchecked for longer, which would lead to more viral particles presented on the host cell surface. The cell would thus be left susceptible for immunosurveillance, offering a possible explanation for the link between bcl-2 expression and better prognosis shown in our study.

Epidemiological studies have reported a lower prevalence of MCV infection in Australian MCC than in the United States or Europe (Sihto et al. 2009; Garneski et al. 2009), which suggests that UVR and MCV infection act through partly separate roles in MCC pathogenesis. Mutations of the \( TP53 \) gene reported in MCC are of the typical UVB type and are restricted to MCV-negative tumours (Popp et al. 2002; Waltari et al. 2011). Bcl-2 is the main regulator of the apoptotic effects of UVB (Knezevic et al. 2007; Bivik et al. 2006). When \( TP53 \) is mutated by UVB it no longer exerts its apoptotic effect via bax and other pro-apoptotic factors. This means that the antiapoptotic effects of bcl-2 are left unsuppressed and the normal UV induced apoptosis is not promoted, leading to a predisposition for skin cancer. In this study, no differences in bcl-2 expression were seen according to either the locations of the tumours, indicating UV exposure, or MCV status. Bcl-2 expression does not differentiate the MCV-specific subgroups of MCC like p53 does.

**CHROMOSOMAL ABERRATIONS AND RB1 HYPERMETHYLATION (STUDY V)**

Study V was conducted to study the chromosomal imbalances of MCV-negative tumours in order to find hints about the possible molecular pathways disrupted in their pathogenesis. ACGH analysis revealed that the genomic profiles of MCC tumours differed distinctly according to MCV status, including frequent losses of the \( RB1 \) locus in MCV-negative tumours. Taking into consideration the importance of RB sequestration in the pathogenesis of MCV infected tumours, we wanted to study the regulation of RB functions further. Thus, \( RB1 \) promoter methylation was studied with the MLPA method and information on RB expression was included in the analyses.
MCV-negative tumours presented with significantly more copy number losses than MCV-positive tumours. Similar findings of increased numbers of losses in the absence of MCV infection have been previously reported (Paulson et al. 2009). Although high frequencies of genomic aberrations have been linked to worse prognosis and larger tumour size of MCC, we did not find a connection between frequent losses and survival (Larramendy et al. 2004; Van Gele et al. 1998). Neither was there a significant association between viral status and survival.

Losses of 11p were significantly more frequent in MCV-negative tumours, which has not been reported previously. The amplification of 1p34.3, which contains the proto-oncogene \textit{L-Myc}, was seen in three MCC tumours, all of which were MCV-negative. The high-grade amplification was first identified in MCC tumours by Paulson and colleagues (Paulson et al. 2009). Since less than half of the tumours presenting with the amplification (4 out of 9) in their material were MCV-negative, it is not restricted to MCV-negative tumours (Paulson et al. 2009).

MCV integration to the MCC genome does not lead to aberrations or mutations of the nearby genetic material (Sastre-Garau et al. 2009). It is suggested that increased chromosomal instability would be an evolutionarily compulsory alternative in MCV-negative MCCs when viral oncogenes could not be depended on. Another explanation is suggested in the hit-and-run hypothesis (Houben et al. 2010b), according to which the genetic material of MCV is gradually lost in some of the tumours, and the cells undergo mutation to retain the tumour progression even without LTA expression. This would then explain the increased genomic instability and the differences detected in the clinical behavior. The reasons behind the better prognosis of MCV-positive MCC reported in various large scale studies still remains controversial.

The primary viral oncogene of MCV, the LT, functions most importantly by sequestering the RB (Shuda et al. 2008). However, Sihto and coworkers found that RB expression was lost only in MCV tumours that did not express LT (Sihto et al. 2011). In addition, a 2.4 fold
increase in the transcription of RB1 has been reported in MCV-positive tumours (Harms et al. 2012). It is thus likely that the inactivation of RB also plays a role in the pathogenesis of MCV-negative tumours, albeit through a different mechanism than in MCV-positive tumours. The MCC tumours included in this study represent a part of the material employed in the study by Sihto and colleagues (Sihto et al. 2011). RB expression was distinctly more common in MCV-positive tumours and was associated with better survival.

22% (6/27) of our MCC samples presented with a heterozygous deletion of the RB1 locus. The respective percentage in the literature varies between 21-40% (Larramendy et al. 2004; Paulson et al. 2009; Van Gele et al. 2002). 83% of RB1 deletions were detected in MCV-negative tumours in our material, whereas Paulson and colleagues reported a percentage of 67% (Paulson et al. 2009). Although no statistical significance was reached in either of the studies, RB1 losses are somewhat more common in the absence of virus infection. Other mechanisms behind RB inhibition in MCV-negative tumours remain in large part unclear. No immunohistochemical differences between MCV-positive and negative tumours have been detected in the expression of regulatory proteins or phosphorylation of RB (Houben et al. 2010a; Sihto et al. 2011).

This study is the first to determine the methylation status of the RB1 promoter in MCC. We used the MLPA method because of its merits in analyzing DNA extracted from paraffinised tumour samples. All of the analysed MCC samples presented with a low-grade methylation of the promoter area spanned by two consecutive probes located in the vicinity of the ATG transcription start site. The methylation pattern was distinctive to MCC tumours compared to normal skin and blood sample references. More proof of a distinctive methylation pattern is offered by the fact that all of the tumour samples showed a similar spectrum. The methylation distribution did not differ according to the viral status, RB expression or survival. Unfortunately, the tumour percentages of our samples were not uniform, which makes it impossible to analyse the small difference in methylation percentages seen between samples. In addition, the differences in tumour percentages prevent the interpretation of possible heterozygosity or strand specificity of the methylation.
It is difficult to deduce the actual effect that the low-grade methylation has on RB1 transcription and consequently molecular pathogenesis of MCC. It is important to note that many of the most important transcription factors of RB1 bind near to the transcription start site methylated in all of the MCC samples (Gill et al. 1994; Stirzaker et al. 1997). It is feasible that RB1 promoter methylation, together with the heterozygous deletion of the RB1 locus, partly explain the losses in RB expression seen in MCV-negative tumours. The effects of inactivating RB1 mutations and transcriptional regulators such as imprinting and miRNAs remain to be clarified.

FUTURE PROSPECTS

MCV DNA sequences are found in buffy coats of peripheral blood leukocytes, suggesting that the lymphocyte pool might act as a reservoir for latent MCV (Shuda et al. 2009; Pancaldi et al. 2011; Mertz et al. 2010). Immunodeficiencies can lead to reactivation of polyomaviruses and acute infection (Sundsfjord et al. 1999; Palazz et al. 2012). Acute manifestations of the MCV infection have not been reported, but 9.5% of the peripheral blood mononuclear cells of HIV-positive non-MCC patients were found to contain MCV DNA, while none was detected in those of healthy controls (Shuda et al. 2009). These findings suggest that immunosuppression could play a part in reactivation of latent MCV. The mechanisms keeping the virus at latency are poorly known. Similarly, the mechanisms by which immunodeficiencies interact with MCV infection remain unclear. Do disturbances in immune functions lead to an active MCV infection or is the predisposition for MCC caused by disturbances in tumour surveillance?

Viral replication is prevented in MCC by the truncating mutations of the LTA. Continued viral replication in the absence of LTA truncation has been suggested to lead to DNA damage responses and correspondingly increased antitumour activity. In immunocompromised patients, tumour surveillance is compromised, which leaves more time for mutagens such as UV exposure and chronic inflammation to truncate the LTA.
Future studies in the area of immunodeficiencies should address the mechanisms underlying the predispositional effects for MCC in order to find more precise treatment options. Even more challenging will be to find the mechanisms of interplay between the immunosuppressive effect of UVR exposure and immunosenescence, combined to other immunodeficiencies.

New immunologic treatment options have recently arisen in the field of MCC therapy. Interferon-α restricts MCC tumour cell proliferation in vitro, but its use in vivo led to adverse effects that forced to end the treatment (Krasagakis et al. 2008; Biver-Dalle et al. 2011). At the moment, intralesional interferon-β treatment is the most promising of possible interferon therapies (Miller et al. 2013). Administering interleukin-2 and interleukin-15 in vitro activates the immune responses in MCC tumours and diminishes the tumour cell count (Dowlatshahi et al. 2013). In a large international multi-center study, MCC patients are treated with a combination of systemic interleukin-2, an angiogenesis inhibitor and paclitaxel (U.S. National Institute of Health 2014a). No results have been published so far. Ongoing clinical trials also include plasmid DNA therapy targeting the interleukin-20 gene (U.S. National Institute of Health 2013).

Advances in the treatment of melanoma serve as groundbreakers that lead to new therapy trials of MCC (Miller et al. 2013). Autologous T-cell treatment has proven effective in metastatic melanoma (Rosenberg et al. 2011) and a phase I/II clinical study that uses MCV LT-specific CD8+ lymphocytes is currently recruiting MCC patients (U.S. National Institute of Health 2014b). Blocking the PD-1/PD-L1-pathway has provided promising results in melanoma (Topalian et al. 2012, Brahmer et al. 2012). An ongoing clinical trial utilizes an anti-PD-L1 molecule in the treatment metastatic MCC (U.S. National Institute of Health 2014c). In another recruiting clinical trial, MCC patients are treated with CTLA-4 protein blocker ipilimumab which is already FDA-approved in the treatment of melanoma (U.S. National Institute of Health 2014d, Hodi et al. 2010).
The observations in this thesis will hopefully serve in practice. The epidemiological studies here amount to the recommendation that skin growths of statins users and autoimmune patients should be thoroughly examined — and even looked for — during routine controls of the basic disease. The inclusion of bcl-2 immunohistochemistry to the diagnostic immunohistochemical panel of MCC could give extra information when determining the needed imaging studies and surgical margins in challenging MCC cases. As for the molecular studies, our results reinforce the role of RB in the pathogenesis of MCV-negative tumours and will pave way to further innovations by others as well as ourselves.

Our future projects include the determination of MCV status and TIL spectrum of MCC patients with a history of AD or statin use. The case-control protocol we designed for study II will be utilized to evaluate the effects that biological disease-modifying antirheumatic drugs pose on MCC risk. We will also continue to study the molecular biology of MCV-negative MCC to find out the characteristic mechanisms behind the pathogenesis of this MCC subgroup. Next generation sequencing and microRNA profiling of MCV-negative tumours could provide us with fresh clues on the involved signal pathways.
CONCLUSIONS

I MCC diagnosed before the age of 50 years is even rarer than previously reported. Unusually young MCC patients are frequently immunocompromised and MCV infected.

II Autoimmune diseases in general lead to a predisposition for MCC, although the effects of therapeutical agents could not set apart with our protocol. Specifically rheumatoid and diabetic conditions increase the risk of MCC.

III The incidence of MCC is increased among patients using statins. The excess of MCCs is observed in atypically young MCC patients with a significant downward trend towards older age groups.

IV Bcl-2 expression is associated with a lower disease stage at the time of diagnosis and a better prognosis of MCC. Bcl-2 expression does not correlate with MCV status of the tumours, age of the patients or location of the tumour.

V MCC tumours present with increased numbers of copy number losses in the absence of MCV infection. Deletions of the $RB1$ locus, amplifications of 1p34.3 and losses of 11p are seen mainly in MCV-negative tumours. Loss of RB expression is associated with $RB1$ deletions and poor survival. Distinctive methylation of the $RB1$ promoter at CpG islands close to the ATG start codon is consistent despite viral status, RB expression or survival.
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