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Population based and clinical epidemiologic studies on different forms of skin cancer

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Dedications

I would like to dedicate this dissertation to the following people who have played a crucial role in my academic journey:

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List of Abbreviations

AIC	Akaike information criterion
AK	Actinic keratosis
ATM	Ataxia telangiectasia mutated
BCC	Basal cell carcinoma
CTLA	Anti-cytotoxic T-lymphocyte-associated antigen
CTLA-4	Anti-cytotoxic T-lymphocyte associated protein 4
DCR	Disease control rate
DNA	Desoxyribonucleic acid
EV	Epidermodysplasia verruciformis
HIV	Human immunodeficiency virus
HPV	Papillomavirus infection
HR	Hazard ratio
hTERT	Transcription of human telomerase reverse transcriptase
ICIs	Immune checkpoint inhibitors
irAE	immune-related adverse events
KC	Keratinocyte cancer
L	Likelihood function
LL	Log likelihood function
MAML1	Mastermid-like 1
ML	Maximum likelihood
NMSC	Non-melanoma skin cancer
OR	Odds ratio
OS	Overall survival
OTRs	Solid Organ Transplant recipients
PD-1	Programmed cell death protein 1
PFS	Progression-free survival
PR	Partial response
pRb	Retinoblastoma tumor suppressor
RECIST	Response evaluation criteria in solid tumours
RNA	Ribonucleic acid
SCC	Squamous cell carcinoma
SE	Standard error
SPSS	Statistical Package for Social Sciences
TGFβ	Transforming growth factor β
Treg	CD4+CD25 regulatory T cell
UV	Ultraviolet

1 INTRODUCTION

1.1 Main focus of my PhD

Many questions arise in the practice of medicine that lead to research projects. These clinical studies usually deeply understand the clinical problems under investigation. However, the lack of the most appropriate statistical approach is not uncommon.

The use of appropriate statistical methods is crucial in the medical research field to ensure robust and accurate analyses. Despite the complexities, statistical methods are central to deriving meaningful insights from data and in informed decision making. By incorporating rigorous statistical approaches, we can improve the reliability and validity of research findings, ultimately advancing medical knowledge and practice. During my PhD program, I attempted to bridge this gap by emphasizing the importance of balancing clinical understanding with appropriate statistical methods in the field of dermatological oncology. I have endeavored to identify the most appropriate statistical approaches for each of two research projects. This thesis represents the culmination of comprehensive analyses that integrates two distinct approaches (logistic regression analysis, Cox regression with time dependent covariates) to examine the data. In this way, I hope to contribute to a more comprehensive and rigorous scientific understanding in the field of oncological dermatology.

1.2 **Study number 1:** Modifiable Risk-factors for Keratinocyte Cancers in Australia: A Case-control Study (1)

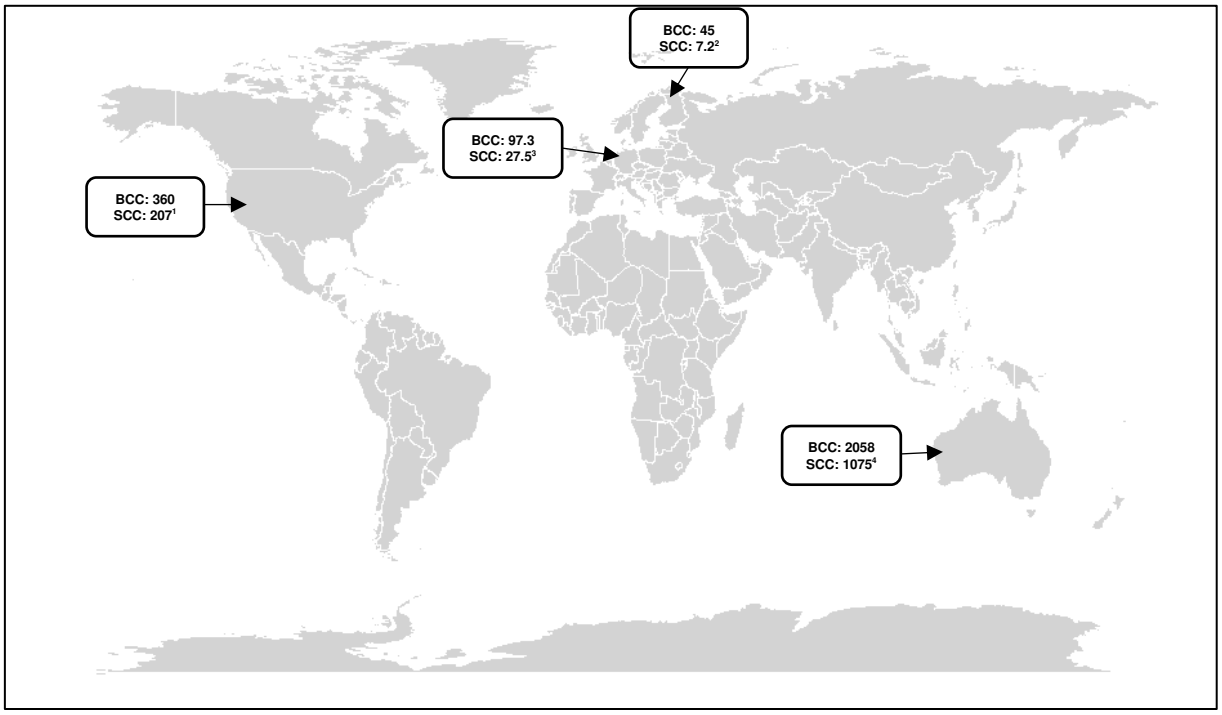
Keratinocyte cancer (KC) or non-melanoma skin cancer (NMSC) is a tumor resulting from the malignant transformation of keratinocytes, cells that make up the epidermal layer (2-4). KC comprise mainly basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and some rare skin tumors (5). BCC accounts for three-quarters of registered cases of KC (4), it is a slow-growing malignant epidermal tumor with low risk of metastasis (6, 7). SCC represents the second most common KC (8), being a more aggressive tumor, with a greater probability

of metastasis and eventual death (9). BCC and SCC are an important health problem in medical practice (10). Globally they causes 5,400 deaths per month, which are mostly related with SCC (10). In addition, KC adds significant morbidity, mainly when they occur in highly visible areas such as the head, neck and face (11, 12).

1.2.1 Epidemiology of Keratinocyte cancer

KC is the most frequent malignancy among white-skinned individuals throughout the world (2, 13), accounting for approximately 30% of all cancers (14) and 90% of all malignant skin tumors (2, 10, 12, 15, 16). The worldwide incidence of KC is constantly increasing (17). with reported annual incidence of KC between 3% and 8% (10, 12, 18, 19). Rising incidence rates of KC have been recorded in Europe (17) and Australia, which is the most common type of cancer in Australian population (18, 20-22). Approximately one-third of Australians develops KC yearly (20, 21, 23, 24). (**Figure 1**). The 2002 Australian National Skin Cancer Survey reported an age-standardized incidence rate of 1288/100,000 for BCC and 593/100,000 for SCC. Compared with the survey conducted in 1985, there was an increase in the incidence of BCC in people over 60 years of age, and an increase in SCC among people over 50 years (25). These temporal trends in KC may be due, at least partly, to the increase of the ascertainment through screening or better improvements in registration, or higher levels of exposure to ultraviolet (UV) radiation over time (26). Additionally, Australia has a marked North to South gradient with extreme incident rates recorded in Queensland (27, 28)

Figure 1. Incidence per 100.000 of squamous cell carcinoma and basal cell carcinoma stratified by countries

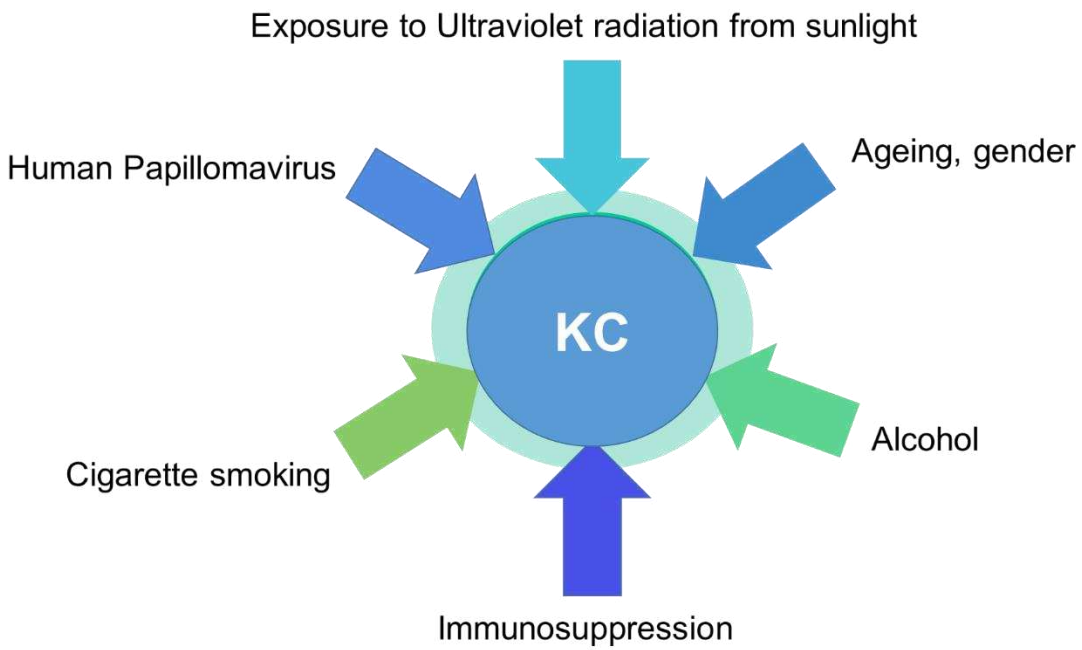


Source: Map: R software, package "maps", version 4.1. [1]Muzic et al. Mayo Clin Proc. 2017 Jun;92(6):890 (29); [2]Hannuksela et al. Arch Dermatol. 1999. 135:781 (30) and [3]Krebs in Deutschland. Robert Koch Institut 2013. [4]Buettner PG. et al. International Journal of Cancer 1998.78:587 (28)

1.2.2 Risk factors:

Although exposure to UV radiation (from sunlight, UV-A therapy, or tanning booths) is recognized as the most crucial factor in the development of KC (31, 32), this factor, however, does not fully explain why some people develop skin cancer, and others do not. Several studies have found other modifiable and un-modifiable risk factors for KC (2, 33); including aging, immunosuppression (14), increased alcohol intake (34), cigarette smoking (23, 35, 36), alterations in plasma lipids and lipoproteins (37), ionizing radiation, contact with chemicals, prior history of an inflammatory lesion, dermatitis, long-established wounds or ulcers, genetic susceptibility to UV-induced carcinogenesis (10), diet (34), and human papillomavirus infection (HPV) (15, 16, 38, 39) (**Figure 2**).

Figure 2: risk factors related with keratinocytes cancers.



1.2.2.1 Ultraviolet radiation exposure:

Exposure to sunlight and artificial UV radiation are well-established environmental risk factors for KC (15, 23, 37, 40-42). This explains why KC has a predilection for areas of direct sunlight exposure (3). UVA/UVB radiation induces direct damage to DNA and RNA by triggering covalent bond formation between adjacent pyrimidines (43), resulting in the generation of mutagenic photoproducts such as cyclopyrimidine dimers and pyrimidine-pyrimidine adducts (9, 43, 44). UV radiation also induces alteration of the repair pathways, induction of oxidative stress, activation of the inflammatory process, and suppression of antitumor immunity (10)

The risk of developing KC is highest among residents of areas with high sun exposure who are susceptible to UV radiation, such as fair skin, eye and hair color, or inability to tan (44). In case of BCC, intermittent sun exposure, especially in childhood, and intensive tanning in the context of leisure activities are associated with the risk of BCC (44). In the case of SCC, cumulative lifetime sun

exposure is of particular importance (8, 45). SCC is more common among people who work outdoors, which are exposed to UV radiation (9).

As KC is related to individual skin tanning behaviors, lifestyle modifications towards skin health protection, like avoiding tanning beds and using UV protection with clothing, sunscreen, and sunglasses, are very important for effective preventive actions (46). In Australia, campaigns to reduce population sun exposure have been implemented to reduce the incidence of keratinocyte cancers (47); however, current data suggest that incidence rates have been increasing in all populations, including Australia (47, 48)

1.2.2.2 Age:

Age is another factor that contributes to the increasing incidence. The underlying explanation is that as we age, the body loses its ability to repair and regenerate itself (10). A study showed that the incidence of KC increases significantly over 40 years of age and even doubles from 40 to 70 years (10).

1.2.2.3 Gender:

Epidemiological studies have indicated that KC is more frequent in males than in females (5). Using a population-based approach, Aggarwal et al. found that KC mortality was twice for males compared to females (5, 49). This might be related to the gender differences in sun exposure behavior. Men are more likely to work outside, which exposes them to higher levels of UV radiation (5). Males are generally less inclined than women to use protective measures as sunscreen and hats (5).

1.2.2.4 Immunosuppression:

Immunosuppression is a well-known risk factor for SCC, including primary (genetic) and secondary immunosuppression (organ transplant recipients, human immunodeficiency virus (HIV) infection, and immunosuppressive drugs) (50). Immunocompromised patients have higher rates of SCC, and their tumors behave more aggressively than tumors in the general population (14, 50, 51). KC

is the most common cancer after transplantation (6). Most skin cancers develop 3-5 years post-transplant and 10-15 years earlier than in the general population (50). A meta-analysis showed that KC incidence among transplant recipients was as high as 12.6% (6).

Immunosuppressive medications exert different effects that increase the risk of KC. Calcineurin inhibitors have pro-oncogenic functions explained by enhancement of tumor angiogenesis and invasiveness, suppression of immunological clearance of malignant cells, reduced apoptosis, and UVB-induced DNA damage repair (50). Regarding other immunosuppressive therapies, a case-control study found that Azathioprine increased twice the risk (odds ratio [OR] = 2.67, 95% confidence interval [CI] 1.23 – 5.76) of developing one cutaneous SCC compared to other immunosuppressive therapies (50, 52)

1.2.2.5 Alcohol:

There are several hypothesized mechanisms by which alcohol may impact skin carcinogenesis. Alcohol is converted into acetaldehyde, a carcinogen that can inhibit the DNA repair system (53, 54). Furthermore, alcohol metabolites can also have a photosensitizing effect, which can increase cellular damage and lead to the generation of reactive oxygen species and related intermediate products (53, 54). In addition, alcohol consumption is linked to a variety of risk-taking health behaviors, including getting sunburnt (48).

Exposure to alcohol and the risk of SC has been investigated in several epidemiology studies, however, the evidence is inconclusive (53, 54). A prospective cohort study of 59.575 post-menopausal women showed that women who consumed seven or more drinks per week had a higher hazard of KC than nondrinkers (55, 56). In a large cohort study of 54.766 participants, no significant association was found between total alcohol consumption and increased risk of BCC and SCC (34, 55). While the relationship may be real, it is also possible that the group of individuals who consumed more alcohol have other unmeasured factors potentially related to skin cancer risk as compared with those who did not drink alcohol (54).

1.2.2.6 Tobacco:

Mechanisms of smoking-induced skin cancer include alteration in the normal balance between cell proliferation, differentiation, and apoptosis. This included delay of DNA repair, accelerates senescence, tumor cell growth, invasion and neovascularization (33). Furthermore, tobacco smoke contains many mutagenic compounds, including oxidants, radicals, and polycyclic aromatic hydrocarbons (57, 58).

Numerous studies have identified smoking as an risk factor for the development of SCC (33, 53). However, the relationship between smoking and the risk of BCC is conflicting. A hospital based case-control study found an association between smoking and SCC (33, 58), with a higher risk for current smokers (OR: 2.0, 95% CI 1.2 – 3.2) than for former smokers (OR 1.9, 95% CI 1.2 – 3.0) (33, 58). Díaz-Corpas et al., found that high-risk SCC was associated with higher cumulative exposure of cigarette (> 20 pack-years OR: 3.63, 95% CI 1.10 – 11.9) (31). Regarding BCC the analyses did not find association between smoking and BCC (33).

1.2.2.7 Human Papillomavirus and skin cancer:

Although the major contributing factor in the development of KC is exposure to ultraviolet (UV) radiation (15, 40)**;** in the last decade a number of viruses have been proposed in the developed of KC, specifically on SCC (15, 16, 38, 39)(15, 59-62). Infections with cutaneous human papillomavirus (HPV), particularly genus β have been the most incriminated virus (13, 14, 16, 40, 61, 63-70). The first evidence for an association of β-HPV and the development of SCC in humans was shown in patients with epidermodysplasia verruciformis (EV) (39, 71), a rare malignant inherited skin disease, characterized by an increased susceptibility of the skin to certain β-HPV type infections (15, 67, 72-74). In these patients, HPV 5 and 8 are present in 90% of their SCC (66, 75). SCC associated with HPV infections have also been described in Solid Organ Transplant recipients (OTRs) (13, 61, 71, 76), where the presence of β-HPV increase the risk of developing SCC (13, 15, 16, 76-79).

Interestingly, the β-HPV viral load decreases during the development of skin cancer, being significantly higher in actinic keratosis (AK), which is considered the premalignant lesion of cSCC (66, 80); suggesting a particular involvement of β-HPV in the early stages of cutaneous oncogenesis (15, 75, 81). Usually, only 1 copy or less of the HPV genome is detected per 1000-10.000 skin cancer cells (14); this finding differs from cervical carcinoma, where α-HPV genomes are detected in each cell (82, 83). This low HPV viral load has made it difficult to confirm the association of β-HPV types with cancer development in the immunocompetent population (15, 84). Cell culture studies suggest that the β-HPV E6 and E7 proteins target anti-apoptotic mechanisms and interfere with pathways that maintain genomic integrity by repairing damage caused by UV radiation, facilitating the accumulation of somatic mutations that contribute to the developed of precancerous lesion, and ultimately to malignant cancer. (85-87). These findings indicate that β-HPV is required only in the initial phase of cutaneous carcinogenesis to introducing/propagating mutations that may destabilize the host cell genome, thereby increasing the carcinogenic potential of the cell, without requiring the continued presence of the viral genome in the later stage to maintain the neoplastic phenotype (the "hit and run mechanism" hypothesis) (15, 83, 88-91).

1.2.3 Justification of the study number 1:

Even so there is a large amount of literature regarding the relationship between sunlight exposure and skin cancer (92-98), the evidence on potential relationships between sunlight exposure, skin constitutional factors, and other potential risk factors on skin cancer risk is limited (34, 99). Different studies suggested that other lifestyle variables could influence the risk of developing KC. Identifying additional risk factors might help develop more effective preventive strategies to reduce the incidence of KC; which might decrease the burden of disease and associated costs, given the high incidence of these malignancies worldwide (2, 5). The present study was designed to elucidate the relationship between environmental and host risk factors in an Australian Caucasian

population. We used binary logistic regression models for the analysis to find variables related to the proposal outcome.

1.3 **Study number 2:** Association between Immune-Related Adverse Events and Survival in 319 Stage IV Study Melanoma Patients Treated with PD-1-Based Immunotherapy: An Approach Based on Clinical Chemistry (100)

Monoclonal antibodies targeting the immune regulatory checkpoint are a novel form of cancer treatment, improving the prognosis of patients with advanced melanoma, increasing survival rates, and improving quality of life (100-105). Immune checkpoints comprise two negative regulatory pathways: cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death 1 (PD-1) or its ligand (104). Both paths are physiologically expressed to mediate control of CD4+ and CD8+ T-cell responses (106), protecting the body from possibly damaging immune responses and maintaining self-tolerance (107, 108). However, tumors can use this mechanism to evade the immune system by activating immune checkpoints and inhibiting T-cell-mediated death (107, 108). Immune checkpoint inhibitors (ICIs) are used to induce an anti-tumor immune response by blocking immune checkpoints which inhibiting T-cell mediated death (106, 107). Thus PD-1 and CTLA-4 inhibitions induce T cell proliferation and reduce the number of CD4+CD25 regulatory T (Treg) cell-mediated immunosuppression and increasing the ratio of effector T cell to Treg cell in the tumor microenvironment (107).

1.3.1 CTLA-4 and PD-1/PD-L1 pathways:

CTLA-4 expression is limited to activated T-cells, whereas PD-1 is expressed on activated T-cells, B-cells, and myeloid cells (106). CTLA-4 is expressed on naive T cells after stimulation and is constitutively expressed on regulatory T cells; CTLA-4 down-modulates helper T cell activity and enhances immunosuppression mediated by regulatory T cells (109). PD-1 is expressed in lymphoid and myeloid

cell; PD-1 ligation suppresses T cell in peripheral tissues, inhibiting T cells proliferation and the production of proinflammatory cytokines (106, 109)

1.3.2 Immune checkpoint inhibitors:

For the treatment of stage IV melanoma, three ICIs are currently approved: the anti-CTLA-4 antibody ipilimumab and the anti-PD1 antibodies nivolumab and pembrolizumab (100, 101, 106, 110, 111). ICIs can be used as monotherapy or in combination (106). Clinical trials in patients with advanced melanoma have demonstrated the superiority of anti-PD-1 antibodies in better disease control rate (DCR) and prolonging both progression-free (PFS), and overall survival (OS) (100, 101, 108, 112-115), and the combination of nivolumab plus ipilimumab seems to improve other outcomes, including better DCR, prolonged PFS and OS (112). In a study conducted by Mason et al., a total of 152 patients with unresectable melanoma who received ipilimumab and nivolumab were evaluated. They reported an overall objective response rate (ORR) of 41% and DCR of 65% (103). Long-term results have also been published. The analysis from the CheckMate 067 trial, which included 945 previously untreated patients with metastatic melanoma, showed improved long-term clinical outcomes with nivolumab (median overall survival (OS) (60 months) and the combination nivolumab-ipilimumab (median OS 45.5 months) (103, 105, 112).

1.3.3 Immune-related adverse events and ICIs

Although ICIs have significantly improved the survival of advanced melanoma patients (111), immune modulation resulting from ICIs can alter immunologic homeostasis and normal self-tolerance and lead to a peculiar spectrum of toxicities known as “immune-related adverse events” (irAEs) (100, 107-109, 111, 113, 116-120). IrAEs are commonly observed in patients treated with ICIs (113).

It could happen in about 90% of patients treated with the anti-CTLA-4 agent and approximately 50-70% of those treated with any antiPD-1 or anti-PD-L1 antibody (108, 109, 121). They can present a wide range of clinical manifestations in terms of toxicity grades, varying from mild to potentially life-threatening (122), as well

as the number and type of organ involved (123). The most commonly affected organs include cutaneous, gastrointestinal, endocrine, pulmonary, renal, and systemic (fever and fatigue) (106, 107, 109, 119, 120, 124), leading to ICIs discontinuation in approximately 10% of patients **(Table 1**) (100, 123). IrAEs could occur at any time, although more likely to occur within the first 3-6 months of ICIs therapy (125). Chronic toxicities can persist months to years after cessation of the ICIs (106), and long-term follow-up is required for patients who have received ICIs (106).

The mechanisms of irAEs are poorly understood; a variety of hypotheses has been postulated, which includes the unbalancing of the immune system induced by immune checkpoint blockade, which breaks the normal self-tolerance outside of the tumor microenvironment, resulting in nonspecific activation of the immune system towards self-peptides and other non-tumor antigens (100, 106, 108, 109, 120). Another possible involved mechanism, specifically from anti-PD-1/PDL-1 inhibitors, is the altered production of auto-antibodies due to a dysregulation of humoral immune (126).

Table 1 Organ affected immune-related adverse events in patients with cancer treated with immunotherapy (101, 106, 107)

Organ	**Disease**
Cardiac	Myocarditis, Pericarditis
Dermatological	Alopecia areata/universalis, Dermatitis herpetiforme Erythema multiforme, Vitiligo
Endocrine	Adenitis, Autoimmune diabetes mellitus, Hyperparathyroidism, Hypogonadism, Thyroiditis
Gastrointestinal	Enterocolitis, Hepatitis, Lymphocytic gastritis, Pancreatitis
Haematological	Aplastic anaemia, Autoimmune haemolytic anaemia Autoimmune neutropenia, Haemophagocytic lymphohistiocytosis
Muscular	Myalgias, Myositis
Neurological	Aseptic meningitis, Encephalitis, Cranial nerve involvement
Ocular	Conjunctivitis, Episcleritis/scleritis, Orbital inflammation Uveitis
Pulmonary	Interstitial lung disease
Renal	Acute tubulointerstitial nephritis, Renal tubular acidosis, Glomerulonephritis

Organ	Disease
Skeletal	Arthralgia, Arthritis, Enthesitis, Fasciitis, Polymialgia rheumatic
Systemic	Antiphospholipid syndrome, Lupus, Sarcoidosis Sjögren syndrome, Systemic sclerosis

1.3.4 Immune-related adverse events and better outcomes

Different studies indicate a potential association between the development of irAEs during immunotherapy and improved treatment outcomes (101, 113, 115, 119, 122, 127, 128). In a study conducted by Matsuo et al., patients with recurrent/metastatic squamous cell carcinoma of the head and neck who were treated with nivolumab and experienced irAEs exhibited significantly higher objective response rates (ORR) and longer progression-free survival (PFS) and overall survival (OS) compared to patients who did not present irAEs (115). A clinical trial reported that patients treated with anti-PD-1 and experienced early onset of irAEs, specifically rash and pyrexia, exhibited an enhanced tumor response and longer PFS (120, 129). A systematic review of 27 studies reported an association between the presence of vitiligo with PFS (HR: 0.51) and OS (HR: 0.25) (109). Eggermont et al. demonstrated an association between irAEs and prolonged recurrence-free survival (RFS) (113). However, in contrast to previous findings, additional evidence has failed to establish a correlation between irAEs and the therapeutic benefits of immunotherapy in terms of ORR and OS (130, 131)

To date, the reasons for this potential association between the occurrence of irAEs and patient outcomes remained unclear. Studies suggest that ipilimumab-induced T-cell proliferation and activation may lead to nonspecific immune response activation (107, 132). Other possible mechanisms include the induction of a nonspecific increase in endogenous T-cell response by ipilimumab mediated by dendritic cells or paracrine cytokine stimulation (126) and a generated cross-reactivity between tumor neoantigens and normal tissue antigens (107, 132).

1.3.5 Justification of study 2

Although ICIs have modified the therapeutic landscape for patients with advanced melanoma, some melanomas are inherently resistant to ICIs (111), and those patients do not benefit from these therapies (133). Therefore, identifying biomarkers to identify patients who will benefit from ICIs optimally is an essential topic for the oncology community (128). In this retrospective analysis, we intended to evaluate if the development of irAEs, assessed by changes in normal routine laboratory parameters, correlates with an improvement in higher DCR, PFS, and OS in patients with advanced melanoma treated in a real-world setting. We used appropriate statistical methods to avoid biases arising from differences in the duration of follow-up and the treatment exposure between patients who did and did not develop irAEs (113). We adjusted the analyses also for possible confounders.

2 MATERIALS AND METHODS

2.1 Methodology study number 1: logistic regression model

2.1.1 Logistic regression model definition:

Logistic regression is a popular regression method that extends linear regression models (134-136), used to model binary response variables (137-140).

If we want to evaluate the probability (p) that the event of interest will occur, we can start using linear regression to estimate the coefficients of β_0, and β_1 as follows (134, 137, 138):

$$p(y = 1 | x_i) = \beta_0 + \beta_1 * x_i \quad (1.1)$$

(ref: Jiang Jingmei, Applied Medical Statistic Book, pp 371 Ed Wiley Blackwell, 2022)

This is, however, invalid as p takes values between 0 and 1, while in the linear regression model, we assume that p follows a normal distribution with a bound between -∞ and ∞. To avoid this, we can take the odds of p:

$$\text{Odds}(p) = \frac{\mathbf{p}}{\mathbf{1-p}} \quad (1.2)$$

(ref: Klein Baum D, et al., Logistic regression, A Self Learning Text, pp 18 Ed Springer, 2010)

The odds of an event occurring is defined as the probability that the event occurs divided by the probability that the event does not occur (141). The odds can also be interpreted as the ratio of success (p) to failure (1-p). However, the odds also have the limitation that their values are always positive and range from 0 to +∞ (142). To circumvent this, we can take the log of odds, with is called logit. By transforming the probability or the odds into logits, the range of values is extended to]-∞ - ∞[

$$\textbf{Logit(p)} = \ln\left(\frac{\mathbf{p}}{\mathbf{1-p}}\right) \quad (1.3)$$

(ref: Backhaus K, et al., Multivariate Analysis an Application-Oriented Introduction, pp 272 Ed Springer Gabler, 2021)

With the transformation of probabilities into odds and logits, we obtain the logistic regression model (134, 137, 139), in which the logit function is modeled rather than the binary outcome (142).

$$\text{logit}(p) = \ln\frac{\mathbf{p}}{\mathbf{1-p}} = \beta_0 + \beta_1 * x \quad (1.4)$$

or

$$\text{probability: } p = \frac{\mathbf{e^{\beta 0 + \beta 1 x}}}{\mathbf{1 + e^{\beta 0 + \beta 1 x}}} = \frac{\mathbf{1}}{\mathbf{1 + e^{-(\beta 0 + \beta 1 x)}}} \quad (1.5)$$

(ref: Backhaus K, et al., Multivariate Analysis an Application-Oriented Introduction, pp 272 Ed Springer Gabler, 2021)

where β_0 and β_1 are unknown parameters that must be estimated (137) and p is the probability. In addition, in terms of odds, they can be expressed as:

$$\text{Odds: odds}[\mathbf{p}(x)] = \mathbf{e^{\beta_0 + \beta_1 x}} \quad (1.6)$$

$$\text{Logit: logit}[\mathbf{p}(x)] = \beta_0 + \beta_1 x \quad (1.7)$$

(ref: Backhaus K, et al., Multivariate Analysis an Application-Oriented Introduction, pp 273 Ed Springer Gabler, 2021)

In this equation the intercept term β_0, is the log of the odds of an event when all covariates are set to zero, and β_1 represent log-odds ratios for change of 1 on the respective scale (142). The exponentials of the coefficients β_1 associated to the independent variables are interpreted as the odds ratio of presenting the event (134, 142). An OR equal to 1 means that the odds of the event in 1 group are the same as in another group; there is no difference. An OR greater than 1 indicates that one group has a higher likelihood of having the event compared to the reference group. Finally, an OR less than 1 means that one group has a lower likelihood of having an event than the reference group (139, 142).

2.1.2 Log-likelihood function:

Logistic regression uses the method of maximum likelihood (ML) to obtain the parameter estimates rather than the method for least squares used in linear regression models (134). The ML method yields values for the unknown parameters that maximize the probability of obtaining the entire observed set of data (134, 137, 138).

The likelihood function (L), is constructed with reference to the binomial probability function and the multiplication rules of probability for independent events (134).

$$L(\beta) = \prod p(x_1)^{y_1} (1- p(x_1))^{1 - y_1} \tag{1.8}$$

$$= \prod \left[\frac{e^{\beta_0+\sum\beta_j x_{ij}}}{1+ e^{\beta_0+\sum\beta_j x_{ij}}}\right]^{y_1} \left[\frac{1}{1+ e^{\beta_0+\sum\beta_j x_{ij}}}\right]^{1 - y_1} \tag{1.9}$$

(ref: Kleinbaum D, et al., Logistic regression, A Self Learning Text, pp 114 Ed Springer, 2010)

(ref: Jiang Jingmei, Applied Medical Statistic Book, pp 375 Ed Wiley Blackwell, 2022)

(ref: Hosmer D et al., Applied Logistic Regression, pp 8 Ed Wiley, 2013)

The formula describes the joint probability for the cases and the non-cases (143), where $p(x_i)$ is the conditional probability of success for i_{th} subject given x_i, and 1-$p(x_1)$ is the conditional probability of the i_{th} subject has not success given x_i (138). By taking the logarithm transformation, we get the log-likelihood function (LL) (134):

$$\ln L(\beta) = \sum_{i=1}^{n} y_i \ln\left[\frac{e^{\beta 0+\sum\beta_j x_{ij}}}{1+ e^{\beta 0+\sum\beta_j x_{ij}}}\right] + \sum_{i=1}^{n}(1 - y_1)\ln\left[\frac{1}{1+ e^{\beta 0+\sum\beta_j x_{ij}}}\right] \tag{1.10}$$

(ref: Kleinbaum D, et al., Logistic regression, A Self Learning Text, pp 114 Ed Springer, 2010)

(ref: Jiang Jingmei, Applied Medical Statistic Book, pp 375 Ed Wiley Blackwell, 2022)

Next, it is necessary to find the values of β that maximize the log-likelihood function. A Newton-Raphson iterative method is generally used for solving the equation (134). As the logarithm of a probability is negative, the LL function can only assume negative values. The maximization of LL, therefore, means that the value of LL comes as close as possible to the value 0. LL = "0" would result if the probabilities of the observed outcomes were all "1" and thus the probabilities for the non-observed outcomes were all 0 (134).

2.1.3 How calculate the significance values of a logistic regression model:

After estimating the logistic regression parameters using the maximum likelihood estimator, it is needed to evaluate the importance of each of the explanatory variables (140), which are assessed by performing statistical tests of the significance of the coefficients. Several statistics can be used to carry out the assessment, including the likelihood ratio test and Wald test.

2.1.3.1 Likelihood ratio test

The likelihood ratio test (the logarithm of the likelihood ratio) compares the likelihood of the model under investigation (fitted model) with the likelihood of the corresponding 0 model (model without predictor variables) (137).

$$LLR = -\mathbf{2In}\left(\frac{\textbf{Likelihood of the 0 model}}{\textbf{Likelihood of the fitted model}}\right) = -2[\mathbf{In}(\frac{L_0}{L_1})] = -2(LL_0-LL_1) \qquad (1.11)$$

(ref: Backhaus K, et al., Multivariate Analysis an Application-Oriented Introduction, pp 303 Ed Springer Gabler, 2021)

where L_0, is the maximum value for the likelihood function of a null (simple) model and L_1, is the maximum value for the likelihood function of a full model. The fitted model will have all the parameters of interest, and the null (simple) model. The likelihood ratio test statistic is chi-square distributed (144), where the degrees of freedom are equal to the number of parameters in the full model minus the number of parameters in the more simple model (140). The LLR can also be used

to compare whether adding or removing certain explanatory variables in a model leads to a better fit of the model (137, 138)

$$LLR = -2 \ln\left(\frac{\textbf{maximized log-likelihood for the reduced model}}{\textbf{maximized log-likelihood for the full model}}\right) \quad (1.12)$$

(ref: Hosmer D et al., Applied Logistic Regression, pp 13 Ed Wiley, 2013)

(ref: Backhaus K, et al., Multivariate Analysis an Application-Oriented Introduction, pp 304 Ed Springer Gabler, 2021)

2.1.3.2 Wald test

Wald Chi square statistics are used to test the significance of singe logistic regression coefficients (145). It is calculated by dividing each coefficient by its standard error (SE) and squaring. Each Wald statistic is compared using a chi square distribution with 1 degree of freedom (140). Wald test is adequate for larges samples (134, 137).

$$X^2_W = \left(\frac{\beta_1}{SE(\beta_1)}\right)^2 \quad (1.13)$$

(ref: Hosmer D et al., Applied Logistic Regression, pp 14 Ed Wiley, 2013)

2.1.3.3 Measures of goodness of fit:

After performing a logistic model, we need to check how well the model describes the response variable (140), or in other words, if the result produced by the model accurately reflects the true outcome in the data (146). It could be done by the deviance (-2LL).

$$Deviance = -2 \ln\left(\frac{\textbf{likelihood of the fitted model}}{\textbf{likelihood of the saturated model}}\right) \quad (1.14)$$

(ref: Hosmer D et al., Applied Logistic Regression, pp 12 Ed Wiley, 2013)

The deviance is the log-likelihood multiplied by -2. Since LL is always negative, (-2LL) will be a positive. A small values indicate a good fit of the model (137, 147).

Additionally, the accuracy of a logistic regression model can be evaluated by discrimination and calibration (148). Calibration refers to the ability to assign the correct average absolute level of risk, whereas discrimination is the ability of the model to assign the risk of an outcome correctly (146, 148). Calibration is often assessed using the Hosmer-Lemeshow goodness of fit test and the R^2 equivalent for logistic regression (149). Discrimination is assessed by the area under a receiver operator characteristic curve of the logistic regression model (136)

2.1.3.4 Hosmer-Lemeshow test

The Hosmer–Lemeshow test is a popular test used to evaluate the goodness of fit of a model (140). It evaluates whether the logistic regression model is well calibrated so that probability predictions from the model reflect the occurrence of events in the data (142). The method is similar to the chi-square goodness of fit. The Hosmer-Lemeshow test involves grouping the sample into g groups (mostly g=10) based on the percentiles of estimated probability. The test statistic is calculated using the observed and expected counts for both events and no events (140), following a chi-square distribution with g - 2 degrees of freedom X^2 (g – 2) (150).

$$X^2_{HL} = \sum_{g=1}^{G} \frac{(O_g - n_g p_g)^2}{n_g p_g (1 - p_g)} \quad (1.15)$$

ref: Hosmer D et al., Applied Logistic Regression, pp 158 Ed Wiley, 2013)

Where n_g is the number of observations in the g_{th} group, O_g is the number of observations with the outcome of interest among n_g observation and p_g is the average predictive probability of the outcome of interest for the g_{th} groups (134). The Obtaining a significant result on the test would indicate that the model is not well calibrated, so the fit is not good (138, 142).

2.1.3.5 R^2 for logistic regression

The R^2 measures for logistic regression mimic the R^2 measure from linear regression, which gives the fraction of the variability in the outcome explained by the model (142). The Cox & Snell and the Nagelkerke R^2 are two R^2 statistics. Nagelkerke R^2 is an adjusted version of the Cox & Snell R^2 and is often preferred (140, 142).

2.1.3.6 Area under the Receiver Operating Characteristic Curve (AUC)

This curve is obtained by plotting the sensitivity (probability of detecting true-positives) over 1- specificity for a full range of possible cutpoints. It assesses over the ability of a model to assign higher probabilities for the outcome to the subgroup that develops the outcome than to the subgroup that does not develop the outcome (138). The ROC curve ranges from 0 to 1.0, a value of 0.5 means that there is classification is not better than tossing a coin. Values largely below 0.5 indicate that the orientation of the predictor has to be changes.

Values above 0.5 are classified as follows (137) :

< 0.7: not sufficient

$0.7 \leq AUC < 0.8$: acceptable

$0.8 \leq AUC < 0.9$: excellent

$AUC \geq 0.9$: outstanding

2.2 Methodology study number 2: Cox regression model

Survival analysis, or time-to-event analysis, is a set of methods to evaluate the relationship between a predictive value and the occurrence of a prespecified event of interest after a follow-up period (151, 152). Those outcomes are very often presented in medical research. A fundamental principle in survival analysis is that this analysis is the combination of whether the event has occurred and when it has occurred. The Cox regression model is a widely used mathematical model for analyzing time-to-event data (143, 153, 154). Cox regression is a model based on the hazard function, which allows evaluation of the effect of one or more variables with an event of interest occurring over time (152, 153). The Cox regression model usually works with time-fixed covariates or time-invariant covariates whose value remains fixed or unchanged throughout the entire follow-up duration (155, 156). However, a common phenomenon in clinical research is that covariate data are collected repeatedly during follow up (153, 154). Those variables are called “time-varying (or time-dependent),” and they may help us to predict better the subsequent course of the patient than baseline values (153, 156-158). Some examples of time-dependent variables are: periodic blood pressure control, cumulative exposure to medicaments, hospitalization etc. An alternative approach to analyze time-dependent variables is by using the time-varying covariate Cox model. This model is an extension of the Cox proportional hazard model and is specifically designed to account for covariates that can change in value during the follow-up period (156, 159).

2.2.1 Type of time-dependent variables:

Time-varying covariates can be classified as either internal or external (156). An internal covariate is an observation generated by the individual under study over time, and it can also affect the failure process and the failure mechanism (155, 156). Examples of internal variables include the Barthel index, blood pressure, procedural history, and CD4 counts measured throughout the study (153). An external covariate, in contrast, can affect the failure process directly but is not

involved in the failure mechanism (155, 156, 160). Examples of external covariable are age and air contamination as risk factors for asthma attacks (156). Extended Cox models are more appropriate for external covariates (156).

2.2.2 Cox regression model definition:

Before beginning, it is necessary to talk about the hazard function, which is based on the probability that an individual will experience an event within a short time interval, given that he or she has survived up to the beginning of that interval (155). The hazard is the limit of this probability, normalized by the length of the interval, when this length tends to zero. The Cox proportional hazards regression model allows us to estimate the effect of covariates on the hazard function (155). The equation of the Cox proportional hazards model is expressed as follows (143, 155, 158):

$$hi(t \mid X) = h_0(t)\exp[\textstyle\sum_{i=1}^{p} \boldsymbol{\beta}_1 X_1] \qquad (2.1)$$

(ref: Klein J et al., Survival Analysis Techniques for Censored and Truncated Data, pp 244, Ed Springer, 2003)

Where X is a set of predictor variables, β denotes the vector of the regression coefficients, which is estimated using the partial likelihood method (161) and $h_0(t)$ represents the baseline hazard function, which corresponds to the hazard when all predictor variables are equal to 0 (143, 155, 161). This model expresses the hazard at time t for an individual with a given specification of a set of explanatory variables (95). In this model, the hazard for individual i at time t is the product of two quantities (143): the baseline hazard function $h_0(t)$, and the exponential expression to the linear sum of $\beta_1 X_1$. An important feature of the Cox model without time dependent covariates is that the hazard ratio does not depend on time and is constant at all times (158). In other words, the baseline hazard is a function of t but does not involve the X's, whereas the exponential expression involves the X's but does not involve t. (143, 155, 156). Therefore, this model assumes that the hazard ratio for each covariate is constant over time

(proportional-hazard assumption of a constant hazard ratio over time) (158, 162, 163).

Cox proposed a model in which covariables are allowed to vary according to a pre-defined function of time (153, 154). The Cox regression model with time-dependent covariates (also known as the extended Cox model) utilizes a step function approach, which looks at different coefficient values to different time intervals (156, 164), so the follow-up time of each subject is divided into shorter time intervals (156). First, for each time–interval, a separate Cox analysis is carried out using the specific value of the time-dependent variable at the beginning of that particular time window (154). Second, a weighted average of all the time window-specific results is calculated (154). This approach resulted in one HR that can be considered as a weighted average of short-term effects on mortality (154).

The equation of Cox proportional hazards model with time-dependent covariates is expressed as follows (156, 165):

$$h(t, X(t)) = h_0(t)\exp[\sum_{i=1}^{p} \boldsymbol{\beta}_i X_i + \sum_{j=1}^{p} \boldsymbol{\delta}_j X_j(t)] \quad (2.2)$$

(ref: Kleinbaum DG et al., Survival Analysis a Self-Learning Text, pp 249, Ed Springer, 2012)

This model contains also the baseline hazard rate $h_0(t)$, which is multiplied by an exponential function; however, in the extended model, the exponential part contains both time independent predictors (β) and time-dependent predictors (δ) (143). $\beta_i(t)$ denotes the value of the time-dependent covariate at time t, and δ quantifies the effect of this covariate at time t to the hazard of an event at the same time point (156). With time-dependent covariates, the proportional hazards ratio in a model with one time-independent predictor and one time-dependent predictor can be represent as follows (143, 165):

$$HR(t) = \frac{\mathbf{h(t,E=1)}}{\mathbf{h(t,E=0)}} \quad (2.3)$$

$$HR(t) = \exp[\beta(1-0) + \delta(1 * t) - (0 * t)] \quad (2.4)$$

$$HR = \exp(\beta + \delta t) \quad (2.5)$$

(ref: Kleinbaum DG et al., Survival Analysis a Self-Learning Text, pp 252, Ed Springer, 2012)

This formula says that the hazard ratio is a function of time; in particular, if δ is positive, then the hazard ratio increases with increasing time (143). Thus, an important feature of this formula is that the proportional hazards assumption is no longer satisfied when using the extended Cox model (143). Another important assumption of the extended Cox model is that the effect of a time-dependent variable $X_j(t)$ on the survival probability at time t depends on the value of this variable at that same time t, and not on the value at an earlier or later time (143).

2.2.3 Estimation of the regression coefficients

The regression coefficients in the extended Cox model are obtained by maximizing the partial likelihood function L (143). The likelihood describes the joint probability of obtaining the data observed on the subjects in the study as a function of the unknown parameters (β´s). It is called “partial” likelihood because the likelihood formula considers probabilities only for those subjects who fail and not for those subjects who are censored (143).

The partial likelihood is expressed by:

$$L(\beta) = \prod_{i=1}^{D} \frac{\exp[\sum_{k=1}^{p} \beta_k Z_{ik}]}{\sum_{j \in R(ti)} \exp[\sum_{k=1}^{p} \beta_k Z_{jk}]} \quad (2.6)$$

(ref: Klein J et al., Survival Analysis Techniques for Censored and Truncated Data, pp 253, Ed Springer, 2003)

Here it is important to point out that the numerator of the likelihood depends only on the information of the person who experiences the event (166), while the denominator includes information about all individuals who have not yet experienced the event (including some individuals who will be censored later) (166). Thus, the partial likelihood can be written as the product of several likelihoods, one for each of k failure times [116]. Once the partial likelihood is estimated, this must be maximized, which is done by taking partial derivates of

the logarithm of L with respect to each parameter in the model, and then solving using the iterative Newton-Raphson technique (166).

2.2.4 Test to assess the significance of the coefficients β:

Following the fit of the regression model, the next step is the assessment of the significance of the model, two test to evaluate the significance of the coefficient are the partial likelihood ratio test and the Wald test (167).

2.2.4.1 The partial likelihood ratio test:

The likelihood ratio test (LR) is calculated as twice the difference between the log partial likelihood of the model containing the covariate and the log partial likelihood for the model not containing the covariate (167). The likelihood ratio test is chi-square distributed, where the degrees of freedom are equal to the number of categories minus the number of parameters in the model (168). The LR can be expressed as:

$$X^2_{LR} = 2(LL(\beta_k) - LL(\beta_{k-1})) \quad (2.7)$$

(ref: Jingmei Jian., Applied Medical Statistics, pp 419, Ed Wiley, 2022)

Where $LL(B_K)$ is the log-partial likelihood function of the model containing all k independent variables and $LL(\beta_{k-1})$ is the log-partial likelihood function of the model without the jth independent variable (134, 167)

2.2.4.2 The partial Wald test:

The Wald test is obtained by dividing the estimate of the regression coefficient (β) by the estimate of the standard error of the regression coefficient (145). It has a chi-square distribution with 1 degree of freedom (143)

3 RESULTS

3.1 Article 1

“Modifiable Risk-factors for Keratinocyte Cancers in Australia: A Case-control Study “ (1)

CLINICAL REPORT

Modifiable Risk-factors for Keratinocyte Cancers in Australia: A Case-control Study

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Keratinocyte cancer is the most common malignancy in Caucasians. The aim of this study was to investigate risk-factors responsible for development of keratinocyte cancer in Australia. A case-control study was conducted, including 112 cases of squamous cell carcinoma (SCC), 95 cases of basal cell carcinoma (BCC) and 122 controls. Freckling during adolescence (SCC: odds ratio (OR) 1.04, $p<0.01$; BCC: OR 1.05, $p<0.01$), propensity to sunburn (SCC: OR 2.75, $p=0.01$, BCC: OR 2.68 $p=0.01$) and high cumulative sun-exposure (SCC: OR 2.43, $p=0.04$; BCC: OR 2.36 $p=0.04$) were independent risk-factors for both SCC and BCC. This study provides further evidence that a sun-sensitive phenotype and excessive sun-exposure during adulthood contribute to the risk of developing keratinocyte cancer. Wearing a hat, long-sleeved shirts, and sunscreen did not significantly reduce the risk of keratinocyte cancer in this study.

Key words: risk factor; keratinocyte cancer; sunlight; sunscreen; basal cell cancer; squamous cell cancer.

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Keratinocyte cancer (KC) arises from the malignant transformation of squamous epithelial cells comprising the epidermis (1). KC includes basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (1, 2). Although KC rarely causes death (3), surgical excision can cause significant morbidity, especially on highly-visible areas, such as the face, ears and neck (4).

KC is the most common malignancy in Caucasians (2, 5). The incidence of KC has increased worldwide by 3–8% annually (6, 7). Australia has the highest reported incidence of KC (8, 9), with the most extreme incidence rates recorded in North Queensland (10, 11). A population-based study conducted in Townsville between 1996 and 1997 found that the age-standardized incidence rates per 100,000 inhabitants for BCC were 2,058.3 for men and 1,194.5 for women, and for SCC were 1,075.7 for men and 517.7 for women (10, 11).

SIGNIFICANCE

This study examined the complex interplay between environmental and host risk-factors for keratinocyte cancer. The results show that increasing age, lower academic qualifications, freckling during adolescence, solar lentiginous, propensity to sunburn and high-cumulative sun-exposure increase the risk of keratinocyte cancer.

The increasing incidence of KC may be explained mainly by high levels of sun-exposure (7) despite the implementation of campaigns in Australia to induce a behaviour change in favour of sun protection and reduce sun exposure (12–14). However, the complex interplay between sociodemographic and environmental risk-factors and the uptake of the various forms of photoprotection is not fully understood.

Exposure to solar ultraviolet radiation (UVR) is a well-established risk-factor for KC (15). Several studies have found modifiable risk-factors for KC other than UVR (16); including diet (17), alcohol consumption (17), cigarette smoking (18–20), and infection with human papilloma virus (21). However, the individual contribution of each factor is not clear, and data on interactions between sun-exposure, host-factors and other potential risk-factors for KC are limited (22), and may explain some inconsistencies in the published literature (2).

The identification of modifiable risk-factors for KC may lead to more effective preventive strategies to reduce the incidence of KC, particularly in high-risk populations. The present study was designed to elucidate the relationship between environmental and host risk-factors in Caucasian patients from Australia who develop KC.

METHODS

Eligible cases ($n=442$) in this case-control study consisted of adults (18–76 years) from the population of Townsville (latitude 19.3°S), North Queensland, who had an incident of BCC or SCC during 2004 to 2009. Cases were patients who presented for treatment at the Townsville Hospital or the surgeries of local surgeons, a dermatologist and general practitioners in Townsville. Only patients with histological diagnosis of *in situ* or invasive SCC or BCC of at least 5 mm diameter on the body or 10 mm diameter or

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more on the head or neck, were included. Cases were compared with age-matched (±5 years) control subjects recruited from local community groups, service clubs and the neighbours of cases. The community-based controls were residents of Townsville with no self-reported history of skin cancer.

Exclusion criteria comprised: skin types V–VI (23), HIV seropositivity, xeroderma pigmentosum, generalized severe dermatological disease, basal cell naevus syndrome, familial atypical multiple mole-melanoma syndrome, transplant recipients, history of SCC or BCC (for controls), initial excision (for cases), and cytotoxic or immunosuppressive therapy within 12 weeks of recruitment. Subjects were also excluded if they had received any of the following treatments within 4 weeks of recruitment: oral corticosteroids on a regular daily basis; inhaled corticosteroids (beclomethasone ≥1,200 µg/day, fluticasone ≥600 µg/day, or budesonide ≥800 µg/day) and regular use of topical steroids to >20% of the skin surface.

A total of 115 subjects (cases and controls) were ineligible based on the exclusion criteria or could not be contacted, leaving 421 subjects. A further 92 subjects were excluded due to frequency matching (see age matching below), leaving a final total of 329 subjects in the analysis (**Fig. 1**).

All cases and controls who fulfilled the eligibility criteria and provided written informed consent to participate were assessed at the Skin Cancer Research Unit clinic. Clinical evaluation was identical for cases and controls: a doctor conducted a full-body skin examination (excluding buttocks and genitals); the research nurse (MG) recorded phenotypic characteristics including natural hair colour at age 18 years (ascertained using wig samples) (24); skin colour, distribution and extent of freckling on the face, forearms and shoulders of participants during adolescence (participants were shown a freckling chart as in previous studies by the investigators) (24) and distribution of solar lentigines on the shoulders (24).

All participants also completed a self-administered questionnaire at baseline to elicit basic demographic information; daily sun-exposure habits for 5 age intervals (school years to age 17; 18–19 years; 20–29 years and 30–59 years); propensity to sunburn; tanning ability and number of blistering sunburns. Duration of sun-exposure experienced on a typical weekday and weekend was recoded as: <1, 1–4, >4–6 and >6 h/day. To measure cumulative sunlight exposure, the following mid-point values were applied to each category for duration of sun-exposure (<1 h=0.5; 1–4 h=2.5; 4–6 h =5; >6 h=8) on a weekday and weekend. The mid-point values for weekday and weekend sunlight exposure were first summed for each age-period group, then summed across age groups, and finally divided into 3 categories: low, medium, and high (25). Frequency of use (always/usually/sometimes/rarely/never) was documented separately for 3 forms of photoprotection (wearing a hat/long-sleeved shirt/sunscreen) during 5 age intervals, then dichotomized as "frequent" (always/usually) or "rare" (sometimes/rarely/ never). Participants who frequently used at least 2 of the 3 forms of photoprotection were considered "frequent multimodal sun-protection users" (26). Highest academic qualification was recoded as: (*i*) primary and secondary school, and (*ii*) trade certificate or technical/college or university degree.

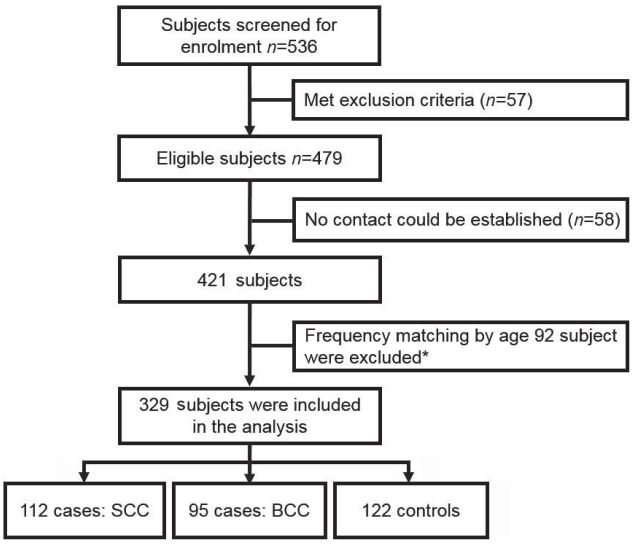


Fig. 1. Flowchart of study participants. BCC: basal cell carcinoma; SCC: squamous cell carcinoma. *Age-matching process is explained in detail in the data analysis section.

Documentation included history of: immunosuppressive conditions, medications, warts, and internal cancers. Lifestyle factors included: smoking, alcohol consumption and dietary intake (typical daily consumption of: bread, cereal, rice and pasta; vegetables and legumes; fruit; milk and dairy products; meat; fish; eggs; nuts; and fluids).

The presence of a KC was histologically-confirmed by obtaining a biopsy of the lesion. Patients who had a single BCC excised were assigned as BCC-cases, whilst patients who had a single SCC excised were considered SCC-cases. Patients with histologically-confirmed BCCs and SCCs excised on the same day were also assigned to the SCC-case group. All slides were reviewed by a specialist in the histopathology of the skin (CG) to ensure that the reported histological diagnosis was accurate.

Ethics approval for this case-control study was granted by the Townsville Health Service District Institutional Ethics Committee (protocol 06/02) and the Human Research Ethics Committee of James Cook University (Approval H2070). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

All participants provided written informed consent prior to data collection. Information collected from participants and their medical records were treated as strictly confidential.

Age matching

Because the mean ± standard deviation (SD) age of cases (60.6 ± 11.4 years) and controls (55.06 ± 11.4 years) was different, frequency matching by age was performed on the original dataset. All cases and controls aged 44–58 years were included in the study. In addition, all cases, but only a random sample of controls younger than 44 years, as well as all controls, but only a random sample of cases older than 58 years, were retained in the final sample of 329 participants (Fig. 1).

Statistical analysis

This project was based on data collected to investigate the effects of environmental factors and human papillomavirus infections on the development of KC. The present analysis was performed on a fixed sample size of 329 participants. Power was assessed ex-post based on the risk of KC according to sun-exposure assuming the effect observed by Iannacone et al. (25). Using the software nQuery, the sample size of 112 cases of SCC and 122 controls had a power of 90% for detecting an absolute difference of 22% (25) in sun-exposure between cases and controls, assuming a type I error of 0.05 (2-sided).

Categorical variables were described using frequencies and proportions; numerical variables were reported as either means ± SD or medians and interquartile range (IQR), depending on the distribution of the data. Normality of the distribution was

assessed by investigating kurtosis, skewness as well as Q-Q plots. Bivariate analyses for both types of KC were performed using χ^2 tests or Fisher's exact test as appropriate. Independent-samples *t*-tests were used to compare numerical variables that were approximately normally distributed, while Mann–Whitney tests were used to evaluate skewed variables.

Binary logistic regression was performed to assess associations between KC status and potential risk-factors. Candidate risk-factors for the multivariate model were selected based on clinical reasoning and statistically significant results in bivariate analyses. Backward selection was used to sequentially remove variables from the model. Crude (simple regression model) and adjusted (multiple regression model) odds-ratios (OR) and 95% confidence intervals (CI) were calculated.

Additional changes in the frequency of sun-exposure and the use of sun-protection across different age intervals were examined. These trends were analysed using the Cochran's Q test. All statistical tests were 2-tailed, and the significance level was set at $p \leq 0.05$. All statistical analyses were performed using IBM SPSS® software, version 23.0 (Armonk, NY: IBM Corp.).

Missing data were assumed to be at random (27) and multiple imputation was used to replace lost data with plausible values, based on the observed data.

Ethics, consent and data protection

Ethics approval for this case-control study was granted by the Townsville Health Service District Institutional Ethics Committee (protocol 06/02) and the Human Research Ethics Committee of James Cook University (Approval H2070). All participants provided written informed consent prior to data collection. Information collected from participants and their medical records were treated as strictly confidential.

RESULTS

This study included 207 (62.9%) cases (95 BCC-cases and 112 SCC-cases) and 122 (37.1%) controls. Age ranged from 27 to 76 years (mean 57 ± 0.5 years) and 53.2% of the sample was male. The demographic, pigmentary and sun-exposure characteristics of participants by case-control status are shown in **Table I**. Compared with controls, both BCC- and SCC-cases were significantly less educated and less likely to develop a tan post-sun-exposure; while being more likely to have light

Table I. Demographic, lifestyle, pigmentary and sun-exposure characteristics of the study population by case-control status (*n* = 329)

	Control (*n* = 122)	SCC (*n* = 112)	*p*-value	BCC (*n* = 95)	*p*-value
Sex, *n* (%)					
Male	56 (45.9)	66 (58.9)	0.05[b]	53 (55.8)	0.15[b]
Female	66 (54.1)	46 (41.1)		42 (44.2)	
Age, years, mean ± standard deviation	55.7 ± 10.1	58.7 ± 10.6	0.03[d]	54.1 ± 10.4	0.24[d]
Highest qualification, *n* (%)					
Primary and secondary school	59 (40.2)	83 (74.1)	< 0.01[b]	63 (66.3)	0.01[b]
Trade certificate/college or university degree	61 (50.8)	29 (25.9)		32 (33.7	
Skin colour, *n* (%)					
Fair	48 (39.3)	74 (66.7)	< 0.01[b]	54 (58.1)	0.01[b]
Olive/medium	74 (60.7)	37 (33.3)		39 (41.9)	
Eye colour, *n* (%)					
Blue/green	63 (51.6)	65 (58.6)	0.29[b]	54 (58.1)	0.35[b]
Brown/hazel	59 (48.4)	46 (41.4)		39 (41.9)	
History of warts, *n* (%)	84 (68.9)	74 (66.7)	0.72[b]	64 (68.8)	0.99[b]
Current warts, *n* (%)	17 (13.9)	24 (21.8)	0.12[b]	25 (26.9)	0.02[b]
Freckling on face, shoulders and forearm in adolescence, median (interquartile range)	7 (0–17)	20 (10–40)	< 0.01[c]	23 (7–40)	< 0.01[c]
Solar lentigines on the shoulders, mean ± standard deviation	32 ± 22)	53 ± 26)	< 0.01[d]	47 ± 26)	< 0.01[d]
Propensity to sunburn (mostly or always burns), *n* (%)	39 (32.0)	76 (67.9)	< 0.01[b]	70 (73.7)	< 0.01[b]
Tanning ability (slow or unable to tan), *n* (%)	15 (12.3)	54 (48.2)	< 0.01[b]	47 (49.5)	< 0.01[b]
Number of blistering sunburns, *n* (%)					
0–2	81 (68.1)	50 (52.1)	0.02[b]	44 (49.4)	<0.01[b]
> 2	38 (31.9)	46 (47.9)		45 (50.6)	
Usually/always used sunscreen in 2+ age-intervals[a], *n* (%)	16 (13.1)	12 (10.7)	0.57[b]	17 (17.9)	0.33[b]
Usually/always wore hat in 2+age-periods[a], *n* (%)	37 (30.3)	51 (45.5)	0.02[b]	28 (29.5)	0.89[b]
Usually/always wore long-sleeved shirt in 2+ age-intervals[a], *n* (%)	45 (36.9)	31 (27.7)	0.13[b]	36 (37.9)	0.88[b]
Accumulated hours sun exposure, *n* (%)					
Low	56 (45.9)	30 (26.8)	0.01[e]	26 (27.4)	0.03[e]
Medium	34 (27.9)	32 (28.6)		38 (40.0)	
High	32 (26.2)	50 (44.6)		31 (32.6)	
Number of cigarettes smoked per day, *n* (%)					
Non-smoker	51 (41.8)	49 (43.8)	0.38[b]	52 (54.7)	0.16[b]
1–10	23 (18.9)	13 (11.6)		32 (33.7)	
11–20	25 (20.5)	30 (26.8)		11 (11.6)	
> 20	23 (18.9)	20 (17.9)			
Alcohol consumption per week, *n* (%)					
Non-drinker	25 (20.5)	33 (29.5)	0.11[b]	24 (25.3)	0.70[b]
1–19 g/week	60 (49.2)	47 (42.0)		42 (44.2)	
>19 g/week	37 (30.3)	32 (28.6)		29 (30.5)	
Other cancers, *n* (%)	11 (9)	13 (11.6)	0.51[b]	15 (15.8)	0.13[b]
Autoimmune diseases, *n* (%)	28 (23)	24 (21.4)	0.78[b]	20 (21.1)	0.74[b]
History of immunosuppressive treatment, *n* (%)	11 (9)	8 (7.1)	0.60[b]	12 (12.6)	0.39[b]
Takes aspirin more than once/month, *n* (%)	40 (34.8)	49 (44.1)	0.15[b]	36 (38.7)	0.56[b]

[a]Age-intervals were divided as follows: schooling 5–17; 18–19 years; 20–29 years; 30–59 years. [b]*p*-value of χ^2 test; [c]Mann–Whitney test; [d]T-test independent variables [e]Linear-by-Linear Association test.

Table II. Binary logistic regression analysis of risk factors for keratinocyte cancer ($n = 329$)

Variable	Squamous cell cancer, $n = 112$			Basal cell cancer, $n = 95$		
	OR	95% CI[a]	*p*-value	OR	95% CI[b]	*p*-value
Sex, male	1.18	0.57–2.43	0.65	1.76	0.89–3.47	0.10
Highest academic qualification:						
Trade certificate/college or university degree	1			1		
Primary and secondary school	2.35	1.19–4.64	0.01	1.73	0.90–3.32	0.10
Skin colour						
Olive/medium	1			1		
Fair	1.76	0.88–3.49	0.11	1.13	0.59–2.19	0.71
Median extent of freckling on face, forearms and shoulders as an adolescent	1.04	1.02–1.07	<0.01	1.05	1.03–1.07	<0.01
Mean density of solar lentigines on the shoulders as an adult	1.02	1.01–1.04	0.01	1.01	0.99–1.03	0.23
Number of blistering sunburns						
0–2	1			1		
>2	1.29	0.65–2.58	0.48	1.39	0.72–2.71	0.33
Propensity to sunburn						
Never–sometimes	1			1		
Mostly–always burns	2.75	1.23–6.16	0.01	2.68	1.23–5.83	0.01
Accumulated hours of sun exposure						
Low	1			1		
Medium	1.50	0.65–3.48	0.34	2.33	1.08–5.01	0.03
High	2.43	1.03–5.74	0.04	2.36	1.04–5.39	0.04

[a]Adjusted for sex, academic qualification, freckling during adolescence, solar lentigines on the shoulders, propensity to sunburn and accumulated hours of sun exposure.
[b]Adjusted for sex, freckling during adolescence, propensity to sunburn and accumulated hours of sun exposure.
OR: odds ratio; CI: confidence interval.

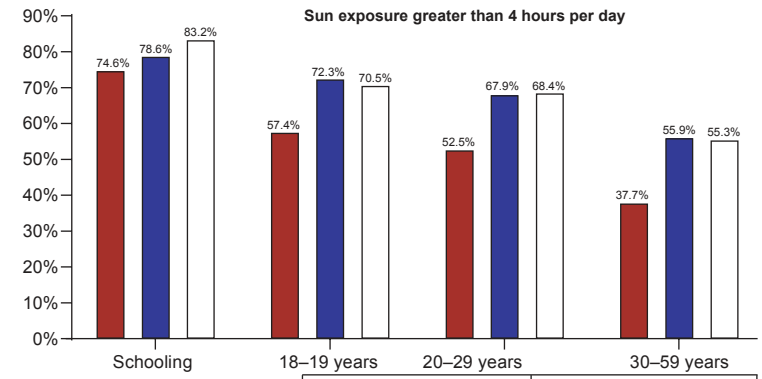
eyes, light colour hair, lentigines, a propensity to sunburn and more freckling on their face.

Risk factors for keratinocyte cancer analysed by binary logistic regression

Using the results from the bivariate analysis, a logistic regression model was generated, which found a significant association between SCC and lower academic qualifications, the presence of freckling, and solar lentigines, propensity to sunburn and a high number of accumulated hours of sunlight exposure. This model explained 39% of the variance in SCC-cases and was a good fit to the actual data (HL $\chi^2 = 9.31$ $p = 0.32$ df = 8) (**Table II**). In addition, a significant association was found between BCC and lower propensity to sunburn, the presence of freckling, and a high and medium number of accumulated hours of sun-exposure (Nagelkerkes R^2: 0.315; HL $\chi^2 = 5.93$ $p = 0.65$ df = 8) (Table II).

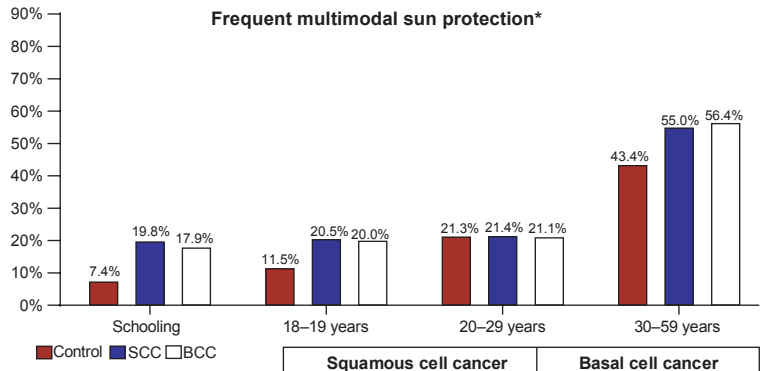
Duration sun-exposure and sun-protection habits

The proportion of cases and controls who spent more than 4 h/day in the sun decreased with age (Control, BCC and SCC $P_{Q\ Cochran} < 0.001$; **Fig. 2**), while frequent-use of multimodal sun-protection (2 of following: wearing a hat/long-sleeved shirt/sunscreen) increased with age in both groups (Control, BCC and SCC $P_{Q\ Cochran} < 0.001$; **Fig. 3**). Sun-exposure of 4+ h/day from 30 to 59 years of age was an independent predictor of BCC and SCC (Fig. 2). More cases than controls used multimodal sun-protection, without conferring any protective benefit against BCC and SCC (Fig. 3). None of the 3 forms of sun-protection (wearing a hat, long-sleeved shirt, and use of sunscreen) by periods-age (period 1: school years to age 17 years; period 2: 18–19 years; period 3: 20–29 years and period 4: 30–59 years) reduced the odds of SCC or BCC, even after adjustment. Conversely, wearing a hat for more than 3 periods was statistically significant related to the risk of SCC (**Table III**). Similarly, long-term use of sun-protection (2–4 age-intervals) did not reduce the likelihood of KC (Table III); since patients with a



	Squamous cell cancer			Basal cell cancer		
	OR	95% IC[1]	p	OR	95% IC[2]	p
Sunlight exposure schooling* (n=329)	1.14	0.51–2.53	0.75	1.86	0.76–4.54	0.17
Sunlight exposure 18-19 years (n=329)	1.63	0.77–3.43	0.20	1.79	0.85–3.79	0.13
Sunlight exposure 20-29 years (n=329)	1.99	0.96–4.14	0.06	1,70	0.85–3.40	0.14
Sunlight exposure 30-59 years (n=327)	2.65	1.30–5.43	0.01	2.74	1.38–5.45	<0.01

Fig. 2. Duration of sun-exposure for cases and controls shown by age intervals ($n = 329$). Sun-exposure greater than 4 h per day during summer or holidays shown by age intervals. OR: odds ratio; CI: confidence interval. [1]Adjusted for sex, academic qualification, freckling during adolescence, solar lentigines on the shoulders, propensity to sunburn and accumulated hours of sun exposure. [2]Adjusted for sex, freckling during adolescence, propensity to sunburn and accumulated hours of sun exposure. *Schooling generally begins at age 5 years and finishes at age 17 years in Queensland, Australia.



	Squamous cell cancer			Basal cell cancer		
	OR	95% IC[1]	p	OR	95% IC[2]	p
Multiprotection schooling* (*n*=329)	3.69	1.37–9.93	0.01	2.55	0.89–7.27	0.08
Multiprotection 18–19 years (*n*=329)	1.84	0.77–4.39	0.22	1.26	0.49–3.21	0.63
Multiprotection 20–29 years (*n*=329)	0.91	0.42–1.98	0.82	0.60	0.26–1.36	0.22
Multiprotection 30–59 years (*n*=327)	1.54	0.81–2.93	0.19	1.47	0.77–2.79	0.24

Fig. 3. Frequent use of multimodal sun-protection by cases and controls, shown by age intervals (*n* = 329). OR: odds ratio; CI: confidence interval. *Use of at least 2 of the 3 sun-protection measures (wearing a hat, long-sleeved shirt or sunscreen). **Schooling generally begins at age 5 years and finishes at age 17 years in Queensland, Australia. [1]Adjusted for sex, academic qualification, freckling during adolescence, solar lentigines on the shoulders, propensity to sunburn and accumulated hours of sun exposure. [2]Adjusted for sex, freckling during adolescence, propensity to sunburn and accumulated hours of sun exposure.

history of skin cancer may have different behaviour with respect to sun protection measures, analyses were also performed omitting information on sun protection after the first skin cancer, however, with the exception of wearing a hat for more than 3 periods, which lost statistical significance, the other results were similar to those of the full cohort (Table SI[1] and Fig. S1[1]). Sunscreen was the least utilized form of sun-protection. Use of all 3 forms of sun-protection increased from 1980 onwards (**Fig. 4**).

[1]https://www.medicaljournals.se/acta/content/abstract/10.2340/00015555-3107

Other risk-factors

History of internal cancers, and dietary intake were similar for both groups (data not shown) and previous autoimmune therapy was not significantly associated with BCC or SCC. No dose-response was evident for number of cigarettes smoked or the duration of smoking and the risk of KC even after adjustment. Likewise, there was also no association between higher alcohol consumption and the risk of SCC or BCC (**Table IV**). No difference in SCC or BCC risk was evident for the different types of alcohol consumed (e.g. beer/sherry/spirits) (data not shown). Although fewer SCC-cases than controls drank wine/champagne (SCC vs. Control 30.4% vs. 52.5%), the risk of KC was not significantly reduced (adjusted-OR 0.68; 95% CI 0.33–1.41, $p = 0.31$).

DISCUSSION

This case-control study found that a high propensity to sunburn increases the risk of KC, and high levels of cumulative sunlight exposure doubled the risk of developing KC compared with those who have low levels of cumulative sunlight exposure. In addition, lower academic qualifications, extent of freckling during adolescence, the presence of solar lentigines on the shoulders during adulthood, and propensity to sunburn were also independent risk-factors for the development of SCC and BCC.

These findings suggest that pigmentary characteristics indicative of a sun-sensitive phenotype and sun-exposure accumulated during adulthood (regardless of childhood

Table III. Bivariate and multivariate analyses of the influence of sun-protection methods on the risk of developing keratinocyte cancer (*n* = 329)

	Control *n* = 122 *n* (%)	Squamous cell cancer (*n* = 112)			Basal cell cancer (*n* = 95)		
		n (%)	Unadjusted model OR (95% CI)	Adjusted model[b] OR (95% CI)	*n* (%)	Unadjusted model OR (95% CI)	Adjusted model[c] OR (95% CI)
Sunscreen use: usually/always by age intervals[a]							
0 age-periods	81 (66.4)	72 (64.3)	1	1	56 (58.9)	1	1
1–2 age-periods	34 (27.9)	34 (30.4)	1.13 (0.64–1.99)	1.17 (0.56–2.46)	31 (32.6)	1.32 (0.73–2.40)	1.06 (0.51–2.21)
3–4 age-periods	7 (5.7)	6 (5.3)	0.96 (0.31–3.00)	0.91 (0.26–3.12)	8 (8.4)	1.65 (0.57–4.82)	0.92 (0.47–1.80)
Hat use usually/always by age intervals[a]							
0 age-periods	49 (40.2)	32 (28.6)	1	1	28 (29.5)	1	1
1–2 age-periods	52 (42.6)	42 (37.5)	1.24 (0.68–2.26)	1.19 (0.56–2.56)	48 (50.5)	1.62 (0.88–2.97)	1.65 (0.81–3.38)
3–4 age-periods	21 (17.2)	38 (33.9)	2.77 (1.38–5.55)	2.62 (1.02–6.25)	19 (20)	1.58 (0.73–3.44)	1.15 (0.46–2.87)
Long-sleeved (L/S) shirt use							
0 age-periods	49 (40.2)	51 (45.5)	1	1	32 (33.7)	1	1
1–2 age-periods	38 (31.1)	39 (34.8)	0.99 (0.54–1.79)	1.06 (0.50–2.26)	35 (36.8)	1.41 (0.74–2.67)	1.49 (0.68–3.28)
3–4 age-periods	35 (28.7)	22 (19.6)	0.60 (0.31–1.17)	0.70 (0.31–1.60)	28 (29.5)	1.23 (0.63–2.39)	1.08 (0.52–2.24)
Number of age intervals with multimodal sun-protection[d]							
0–1 age intervals	94 (77)	83 (74.8)	1	1	69 (73.4)	1	1
2–4 age intervals	28 (23)	28 (25.2)	1.13 (0.62–2.07)	0.91 (0.43–1.93)	25 (26.6)	1.22 (0.65–2.27)	0.80 (0.37–1.73)

[a]Age-intervals were divided as follows: Schooling 5–17 years; 18–19 years; 20–29 years; 30–59 years, schooling generally begins at age 5 years and finishes at age 17 years in Queensland, Australia. [b]Adjusted for sex, academic qualification, freckling during adolescence, solar lentigines on the shoulders, propensity to sunburn and accumulated hours of sun exposure. [c]Adjusted for sex, freckling during adolescence, propensity to sunburn and accumulated hours of sun exposure. [d]Number of intervals in which a participant frequently used at least 2 of the 3 forms of sun-protection (hat/long-sleeved shirt/sunscreen) on a warm sunny day.
OR: odds ratio; CI: confidence interval; L/S: long-sleeved shirt.

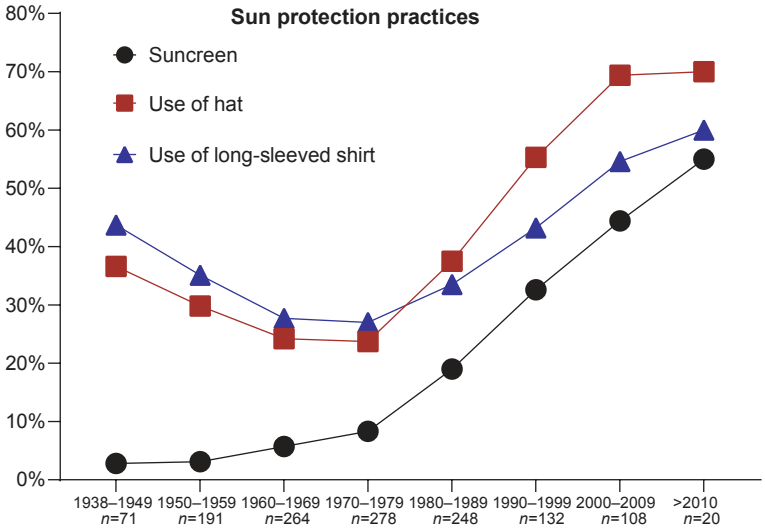


Fig. 4. Proportion of participants who usually/always use sun-protection*, shown by chronological time ($n = 329$). *Use of sun-protection measures by chronological time (wear a hat, long-sleeved shirt or sunscreen). Note that younger participants only contribute data to later time-intervals, whereas older participants contribute data across all time-intervals. Thus a potential bias due to cohort effects or attrition cannot be excluded.

sun-exposure) are important in the development of KC (28–31), suggesting that reducing sun-exposure during adulthood can help prevent KC. These findings are similar to those from a cohort-study of 56,667 women, which showed that sun-exposure during adulthood increased the risk of KC irrespective of childhood UVR-exposure (32), but differ from the case-control study by Iannacone and co-workers, which showed that childhood sun-exposure increased the risk of SCC, but not of BCC (25). Given these conflicting findings, it seems important to clarify whether there are vulnerable periods in life during which sun-exposure is more harmful.

Since sun-exposure represents the most important environmental risk-factor for KC (20) several approaches have been established to reduce exposure, including avoiding direct midday sun-exposure, wearing sun-protective clothing, and applying high sun-protection-factor (SPF) sunscreen (30, 33). Frequent sunscreen-use did not appear to reduce the risk of KC in the present study. This is consistent with a randomized controlled trial that did not show any significant difference in the incidence of KC between "daily sunscreen" and the "no sunscreen" group (34, 35). One plausible explanation is that sunscreen-users stay outdoors longer, merely delaying sunburn (or accumulating a high sub-erythemal dose) rather than preventing over-exposure (36–38). Furthermore, the effectiveness of sunscreen depends on its SPF, the amount applied, application frequency, and the user's skin-phototype (36, 39–41). Some authors have proposed that other physical barriers, such as wearing a hat and long-sleeve shirt, can also help in preventing the harmful effects of UV radiation (35); in the present study, wearing a hat was associated with a significantly elevated risk for SCC. North Queensland is a region with very high insolation, and there is a high frequency of individuals using sun protective measures. This may be the reason for lack of risk reduction by sun-protective practices in our study. Similar findings have been reported previously by others (42).

In order to achieve comprehensive sun protection and reduce the risk of skin cancer, it is necessary to take daily measures to protect oneself from excessive exposure to solar UV-radiation (43). The American Skin Cancer Society (2017) recommends the following primary strategies: (*i*) seek shade when out in the sun, especially in the middle of the day when UV radiation is strongest (10.00–16.00 h); (*ii*) textile protection with appropriate

Table IV. Univariate and multivariate analyses of smoking and drinking status in relation to SCC risk ($n = 329$)

	Control ($n = 122$)	SCC ($n = 112$)				BCC ($n = 95$)			
	n (%)	*n* (%)	OR	95% CI[a]	*p*-value	*n* (%)	OR	95% CI[b]	*p*-value
Duration of smoking									
0 year	51 (41.8)	52 (54.7)	1		0.25	49 (43.8)	1		0.20
1–20 years	30 (24.6)	15 (15.8)	0.53	0.22–1.27	0.16	20 (17.9)	0.49	0.20–1.19	0.11
> 20 years	41 (33.6)	28 (29.5)	0.60	0.28–1.26	0.18	43 (38.4)	0.58	0.28–1.23	0.16
Number of cigarette smoked per day									
No	51 (41.8)	52 (54.7)	1		0.25	49 (43.8)	1		0.18
1–10	23 (18.9)	12 (12.6)	0.56	0.24–1.40	0.21	13 (11.6)	0.45	0.18–1.14	0.09
> 10	48 (39.3)	31 (32.6)	0.57	0.27–1.20	0.14	50 (44.6)	0.60	0.29–1.24	0.17
Duration of drinking									
0 year	12 (9.8)	11 (11.6)	1		0.95	23 (20.5)	1		0.14
1–20 years	14 (11.5)	13 (13.7)	1.03	0.25–4.24	0.97	11 (9.8)	0.30	0.08–1.15	0.08
> 20 years	96 (78.7)	71 (74.7)	1.16	0.39–3.45	0.79	78 (69.6)	0.38	0.14–1.09	0.07
Alcohol consumption									
None	25 (20.5)	33 (29.5)	1		0.95	24 (25.3)	1		0.88
1–19 g/day	60 (49.2)	47 (42.0)	0.92	0.39–2.20	0.86	42 (44.2)	1.24	0.52–2.96	0.63
> 19 g/day	37 (30.3)	32 (28.6)	0.85	0.33–2.21	0.74	29 (30.5)	1.24	0.47–3.26	0.66

[a]Adjusted for sex, academic qualification, freckling during adolescence, solar lentigines on the shoulders, propensity to sunburn and accumulated hours of sun exposure.
[b]Adjusted for sex, freckling during adolescence, propensity to sunburn and accumulated hours of sun exposure.
OR: odds ratio; CI: confidence interval.

clothing (i.e. long-sleeved shirts and long trousers or long skirts) (30, 41); (*iii*) use wide-brimmed hats; (*iv*) use sunscreen with the correct sun protection factor for the skin phototype (individuals with skin phototype I need SPF 50+ protection and those with darker skin phototypes can use SPF 15 products) (41). In addition, the sunscreen should be re-applied after each bath and every 2–3 h during a stay on the beach; and (*v*) avoid the use of tanning beds (44). Other recommended strategies for the prevention of skin cancer would be to reduce the sun-exposure time and outdoor activity during periods of high UV radiation (33, 39), wear sunglasses, parasols and, finally, regular skin self-examination or clinical examination, which enables early detection of skin changes (30). The combination of these approaches has been shown to reduce the burden and reduce the incidence, morbidity and mortality of skin cancer (45, 46).

This study found that a substantial proportion of cases and controls exhibited several risk-behaviours, including spending more than 4 h/day outdoors, and infrequent use of sunscreen, shirts and hats; even though the prevalence of all 3 behaviours increased significantly between 1970 and 2010. The latter is probably a consequence of the mass media campaigns introduced in Australia from 1980 onwards to raise awareness about skin cancer and sun-protection (12, 37). These findings highlight the importance of public health campaigns in encouraging life-long use of sun-protection and promoting regular skin checks (12, 47).

KCs are known to be associated with states of immune perturbation (29, 32, 48, 49). In contrast, we found that cases and controls were similar in relation to use of immunosuppressive therapy. However, as we excluded patients who received immunosuppressive therapy close to the time of diagnosis of KC, the current study was not designed to answer this question.

Study limitations and strengths

The present study has several limitations. Firstly, little data were collected concerning the pattern of sun-exposure (i.e. at midday vs. mornings or late afternoons). Secondly, sun-exposure habits were self-reported. Recall bias is possible, given that case subjects are more likely to be concerned about possible causes of KC, and therefore are more likely to over-estimate their sun-exposure history than controls; and thirdly the size restriction on the keratinocyte cancer included could also may lead a selection bias.

One strength of this study is the availability of data on a large number of potential risk-factors, allowing adjustment of confounding factors. Another strength is that controls were screened for evidence of BCC and SCC by a medical expert to avoid the misclassification of cases and control subjects that might otherwise result from self-reported data. Longitudinal data collected from this cohort may further elucidate the contribution of host and environmental risk-factors to the development of KC.

Conclusion

These findings confirm the increased risk of KC in association with sun-exposure, consistent with other studies. Importantly, this study showed that the frequency of use of sun-protection did not differ significantly between cases and controls. Further investigations are needed focusing on these variables, together with individual susceptibility factors and other potential interacting risk-factors for KC to determine which sun-protection strategies are most effective in preventing KC.

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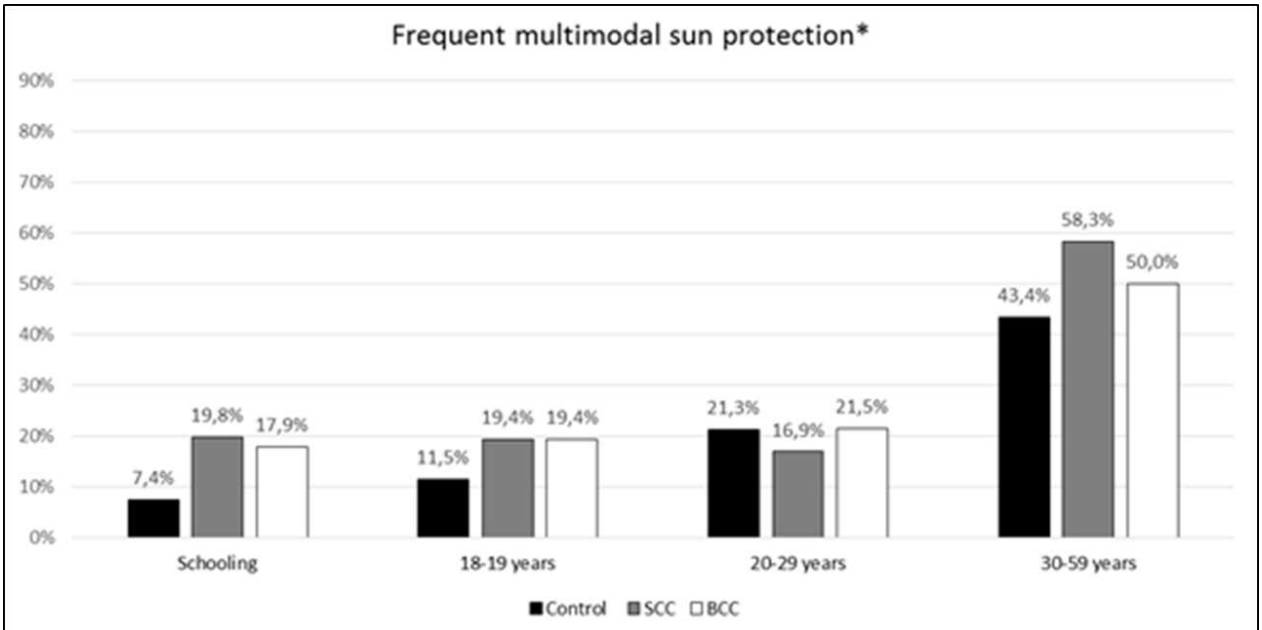
e-Supplements

Table 1 supplement: Bivariate and multivariate analyses of the influence of sun-protection methods on the risk of developing Keratinocyte Cancer, this table includes only the patients who have had at least three age-periods without history of skin cancer (n=290).

	Control n=122	**Squamous cell cancer n=89**			**Basal cell cancer n=79**		
			Unadjusted model	**Adjusted model[1]**		**Unadjusted model**	**Adjusted model[2]**
	n (%)	**n (%)**	**OR (95% CI)**	**OR (95% CI)**	**n (%)**	**OR (95% CI)**	**OR (95% CI)**
Sunscreen use Usually/Always by age intervals[a]							
0 periods	81 (66.4%)	64 (71.9%)	1	1	48 (60.8%)	1	1
1-2 age-periods	34 (27.9%)	21 (23.6%)	0.78 (0.41-1.48)	0.93 (0.42-2.06)	26 (32.9%)	1.29 (0.69-2.41)	1.08 (0.51-2.29)
3-4 age-periods	7 (5.7%)	4 (4.5%)	0.72 (0.20-2.58)	0.80 (0.37-1.71)	5 (6.3%)	1.21 (0.36-4.01)	0.77 (0.18-3.21)
Hat use Usually/Always by age intervals[a]							
0 periods	49 (40.2%)	31 (34.8%)	1	1	23 (29.1%)	1	1
1-2 age-periods	52 (42.6%)	31 (34.8%)	0.94 (0.50-1.77)	1.04 (0.48-2.25)	39 (49.4%)	1.60 (0.84-3.05)	1.66 (0.79-3.48)
3-4 age-periods	21 (17.2%)	27 (30.3%)	2.03 (0.98-4.20)	2.19 (0.91-5.31)	17 (21.5%)	1.73 (0.77-3.87)	1.35 (0.50-3.61)
Long-sleeved (L/S) shirt use							
0 periods	49 (40.2%)	43 (48.3%)	1	1	25 (31.6%)	1	1
1-2 age-periods	38 (31.1%)	29 (32.6%)	0.87 (0.46-1.64)	0.99 (0.45-2.20)	29 (36.7%)	1.50 (0.76-2.96)	1.55 (0.68-3.52)
3-4 age-periods	35 (28.7%)	17 (19.1%)	0.55 (0.27-1.13)	0.68 (0.29-1.61)	25 (31.6%)	1.40 (0.69-2.83)	1.17 (0.83-2.68)
Number of age intervals with multimodal sun-protection[3]							
0-1 age intervals	94 (77%)	68 (76.4%)	1	1	57 (72.2%)	1	1
2-4 age intervals	28 (23%)	21 (23.6%)	1.04 (0.54-1.98)	0.94 (0.43-2.04)	22 (27.8%)	1,30 (0,68-2,48)	0,91 (0,42-1,98)

Figure 1 supplement: Frequent use of multimodal sun-protection by cases and controls, shown by age intervals, this table excludes patients with history of skin cancer



	Squamous cell cancer			Basal cell cancer		
	OR	95% IC	P	OR	95% IC	p
Multiprotection schooling (n=328)	3.80	1.41-10.29	0.01	2.56	0.90-7.22	0.08
Multiprotection 18-19 years (n=323)	1.86	0.77-4.45	0.17	1.27	0.50-3.25	0.62
Multiprotection 20-29 years (n=290)	0.74	0.32-1.71	0.53	0.63	0.27-1.46	0.22
Multiprotection 30-59 years (n=140)	2.71	0.69-10.79	0.16	1.62	0.29-9.00	0.58

3.2 Article 2

“Association between Immune-Related Adverse Events and Survival in 319 Stage IV Melanoma Patients Treated with PD-1-Based Immunotherapy: An Approach Based on Clinical Chemistry” (100)

cancers

MDPI

Article

Association between Immune-Related Adverse Events and Survival in 319 Stage IV Melanoma Patients Treated with PD-1-Based Immunotherapy: An Approach Based on Clinical Chemistry

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Simple Summary: Nivolumab combined with ipilimumab has improved the prognosis of patients with advanced melanoma. However, this therapy is frequently associated with immune-related adverse events. Published data suggested that objective responses rates appear to be superior in patients who developed immune-related adverse events. The primary aim of this study was to evaluate the association between immune-related adverse events and disease control rate, progressive-free survival, and overall survival in patients with stage IV melanoma treated with first-line PD-1-based immunotherapy. In this manuscript, we show that the presence of immune related side effects is related to better overall response and longer survival in patients with advance stage melanoma treated immuno-therapy, suggesting that immune-related adverse events might be a predictive factor of response in those patients.

Abstract: (1) Background: Immune checkpoint inhibitors have improved the prognosis of patients with advanced melanoma. Published data suggested that the objective response rates appear to be superior in patients who developed immune-related adverse events (irAEs). (2) The primary aim of this cohort study was to evaluate the association between irAEs and disease control rate in patients with stage IV melanoma treated with first-line PD-1-based immunotherapy. (3) Among 319 patients, 53% experienced at least one irAE. A higher percentage of patients with irAEs had disease control compared to those without irAEs (69.8% vs. 49.3%). In multivariate analysis, development of grade 3 and 4 irAEs was significantly associated with a protective effect for the outcome primary resistance (OR: 0.40 95% CI 0.23–0.70, $p = 0.001$). The presence of any grade irAEs was significantly associated with longer OS (irAEs grade 1–2 HRadj: 0.61 95% CI: 0.4–0.93, $p = 0.02$, irAEs grade 3–4 HRadj: 0.55 95% CI 0.31–0.99, $p = 0.04$), but not with PFS (irAEs grade 1–2 HRadj: 1.21 95% CI: 0.91–1.79, $p = 0.16$, irAEs grade 3–4 HRadj: 1.14 95% CI 0.83–2.02, $p = 0.24$). (4) The presence of irAEs with laboratorial expression is positively associated with response and OS, suggesting that irAEs might be a predictive factor in this setting.

Keywords: melanoma; immune-related adverse events; response; survival; immunotherapy; anti-PD-1; anti-CTLA-4

1. Introduction

Monoclonal antibodies targeting the immune regulatory checkpoint receptors of anti-programmed cell death 1 (PD-1) and anti-cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) have significantly improved the prognosis of patients with advanced melanoma [1–4]. Currently, three immune checkpoint inhibitors (ICIs) are approved for the treatment of stage IV melanoma: the anti-CTLA4 antibody ipilimumab, and the anti-PD1 antibodies nivolumab and pembrolizumab [1,5,6]. Clinical trials have demonstrated that combined treatment with nivolumab plus ipilimumab and nivolumab monotherapy in patients with advanced melanoma led to better objective response rates (ORR), progression-free (PFS), and overall survival (OS), than treatment with ipilimumab alone [1,7–9]. The 5-year OS rate was 52% in the nivolumab plus ipilimumab group, 44% in the nivolumab group, and 26% in the ipilimumab group [7].

Though the introduction of ICI improved the prognosis of patients with metastatic melanoma, this therapy is frequently associated with immune-related adverse events (irAEs) [8,10–12]. This can be explained as the ICIs play a role in maintaining immune homeostasis and preventing autoimmunity, therefore their inhibition leads to increased activity of the immune system, resulting in a variety of irAEs that resemble autoimmune diseases in their clinical presentation [6,8,10–15]. These irAEs can involve any organ or tissue [16], ranging from mild to life-threatening toxicity [17]. The most commonly irAEs reported are rash, vitiligo, colitis, pneumonitis, hepatitis, thyroiditis, nephritis, and hypophysitis [13,18,19], leading to ICIs discontinuation in approximately 10–20% of patients [16].

A correlation between the diagnosis of severe irAEs and an improvement in PFS and OS in patients receiving ICI has been previously described in diverse tumor entities in the metastatic setting [1,8,13,17,20–22]. In an adjuvant setting, and for melanoma patients, Eggermont et al. were also able to demonstrate a correlation between irAEs and an improvement in recurrence-free survival (RFS) [8]. In contrast to reports based on data from clinical studies, this relationship has not been studied as intensively in real-world data [23]. This work describes the association of the occurrence of immune-related adverse events and improved survival in melanoma using real-world data.

2. Materials and Methods

2.1. Study Design and Data Sources

We conducted a single-center, retrospective cohort study in patients with unresectable stage IV melanoma treated with first-line PD-1-based immunotherapy (pembrolizumab, nivolumab, or nivolumab plus ipilimumab). The German Central Malignant Melanoma Registry (CMMR) was used to initially identify our patients' collective, i.e., patients diagnosed with stage IV melanoma between January 2015 and December 2018. Additional clinical and laboratory data were retrieved from the patients' medical records and further documented in the open-source system Epi InfoTM. Consistency analysis was performed with the database, and patients' medical records from the University Hospital Tuebingen (SAP ISH GUI for Windows) were used to validate and complement the information.

2.2. Population

Patients diagnosed with stage IV melanoma (AJCC 8th) [24], who received first-line PD1-based immunotherapy from January 2015 to December 2018, were included (n = 353). Patients who received first-line monotherapy with ipilimumab (n = 26) and those who had incomplete follow-up data (n = 8) were excluded, thus 319 patients were included in this final analysis.

2.3. Variables

The following clinical data were collected at baseline: age, sex, histological subtype, tumor localization, BRAF, NRAS and c-kit mutational status, presence and localization of metastasis, date and type of systemic therapy received, best overall response (BOR)

to ICI according to Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1) [25], date of progressive disease (PD), and date of patients' last contact or death. The BOR to first-line immunotherapy was defined as the best response—intracranial and extracranial —that patients achieved during the time they were treated [26] and was categorized as either complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). Patients with CR, PR, and SD were considered to have disease control (DC) [26]. Patients with PD as BOR were considered as having primary resistance to ICI, as they did not respond to first-line ICI. Imaging assessment was performed by a radiologist demonstrating the radiological findings during treatment of each patient in the interdisciplinary tumor board.

Due to the retrospective nature of this study, it was difficult to assess the presence of irAEs for which a clinical evaluation is necessary, for example, cutaneous irAEs, since this depends on the documentation of these adverse events in the patients' medical records. Therefore, to improve the data quality, we focused on the irAEs for which objective laboratory values were documented.

The laboratorial parameters used to identify irAEs associated with ICI therapy were retrieved from the central laboratory of the University Hospital Tuebingen between January 2015 and March 2019, allowing us a minimum of 3 months of follow-up after therapy start for the last patient included in the analysis. The laboratorial investigations included in this study were hematological (hemoglobin, platelets, leukocytes, neutrophils, lymphocytes, and eosinophils), hepatobiliary (AST, ALT; GGT, direct bilirubin, indirect bilirubin, and alkaline phosphatase), endocrine (TSH, fT3, fT4, and cortisol) and renal (creatinine, glomerular filtration rate, and blood urea nitrogen). All irAEs were graded according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events version 5.0 (CTCAE) [27]. Patients with liver metastases and elevated liver enzymes at the time of immunotherapy start were excluded, as further elevation of transaminases due to ICI therapy could not be clearly differentiated from liver disease progression.

2.4. Objectives and Endpoints

The primary endpoint was the DC rate, defined as the percentage of patients with DC, and the secondary endpoints included PFS and OS. PFS was defined as the time between the date of therapy start and date of documented PD according to RECIST 1.1; OS was defined as time from start of therapy and patients' last contact or death due to any cause. The data cutoff date for the analysis was March 2019.

2.5. Detectable Effect

The sample size was fixed by the number of patients from the CMMR (n = 319). With this sample size, we had a power of 95% (type I error 0.05 two-sided, chi-square test) to detect group differences in proportions of 0.20 (software PASS 2020). The observed frequency of irAEs (169 with irAEs vs. 150 without irAEs) and the total frequency of patients with DC were used in this calculation.

2.6. Statistical Analysis

We first described the patients' characteristics using the appropriate descriptive statistics according to the type of variables. Qualitative variables were described using absolute and relative frequency. Numerical variables were described as means and standard deviation or medians, and interquartile ranges (IQR) according to the distribution of the data. The normality of the distribution was assessed by investigating skewness and kurtosis as well as QQ graphs, box plots, and histograms. Bivariate analysis was performed by grouping the patients based on the presence or absence of irAEs. Categorical variables were compared using Chi-square tests (or Fisher's exact test for small data sets). Continuous variables were compared between groups (no irAEs, irAEs grade 1–2, and irAEs grade 3–4) using one-way analysis of variance (ANOVA) for normally distributed data or Kruskal–Wallis test for non-normally distributed data.

To test the associations between irAEs and response, i.e., DC vs. PD, we performed a binary logistic regression model. Confounding variables were selected based on clinical reasoning and statistically significant results in bivariate analyses. Crude (simple regression model) and adjusted (multiple regression model) odds ratios (OR) and 95% confidence intervals (CI) were calculated. The goodness of fit was evaluated using the Hosmer-Lemeshow test (HL).

Cumulative incidence (CI) of irAEs was estimated considering death as a competing risk. The proportional sub-distribution hazard model of Fine and Gray was used to analyze the effect of type of therapy, sex, and age on the incidence of irAEs [28,29]. Censored data (PFS and OS) were analyzed using the Kaplan–Meier method, and the log-rank test was used to test differences of survival distribution by groups (irAEs present vs. not present). In addition, a 3-months landmark survival analysis was performed, excluding patients who were lost to follow-up or died in the first 3 months after starting ICI [30]. Univariate time-dependent Cox proportional regression models were used to evaluate the relationships between the outcomes (PFS and OS) and age, sex, histological subtype, BRAF mutation status, number and localization of metastasis, type of immunotherapy, and irAEs as a time-varying variable. IrAEs were categorized as follows: 0 = no irAEs available, 1 = irAEs grade 1–2, and 2 = irAEs grade 3–4. [8,31]. We used a multivariate time-dependent Cox proportional hazard model to assess differences in OS and PFS risk associated with the presence or absence of irAEs. Variables initially introduced in the multivariate survival analyses were all variables associated with PFS or OS in the univariate analyses with a p-value < 0.10 or variables previously identified as risk factors. Interactions between independent covariates were tested in the final models. Hazard ratios (HRs) and 95% CIs were estimated. The proportional hazard assumption was tested for each covariate of the Cox model using the Schoenfeld residual. All reported p values were two sided and the significance level was set at $\leq$0.05. Missing data were assumed to be at random and multiple imputation by chained equations (package "Mice") was applied to handle missing data [32]. All the analyses were carried out using the statistical program for social sciences IBM SPSS software version 26.0 (IBM, New York, NY, USA) and R software version 3.6.

2.7. Ethics Approval

The data were collected as part of routine clinical care in compliance with good clinical practices. The study was approved by the Ethics Committee of the University Hospital Tuebingen (project number 149/2020BO2) and conducted in accordance with the Declaration of Helsinki.

3. Results

The final analysis included 319 patients, with a median follow-up of 24 months (95% CI 19–29). Sixty percent of the patients were men (n = 192). The mean age of the patients at the time of therapy start was 65.5 (SD 14.4, range 19 to 90) years. The BOR to first-line PD-1-based immunotherapy was PD in 39.8% (127 patients), SD in 19.4% (62 patients), PR in 25.1% (80 patients) and CR in 15.7% (50 patients). Table 1 shows the other baseline characteristics of the study population.

3.1. Cumulative Incidence of irAEs

One hundred sixty-nine (53%) patients experienced at least one irAE. Multiple irAEs occurred in the same patient: 1–5 irAEs in 99 patients (31.0%) and more than 5 irAEs in 70 patients (21.9%). The frequency distribution was: hematological (51.1%, 163 patients), renal (28.8%, 92 patients), hepatobiliary (25.4%, 81 patients) and endocrine (24.1%, 77 patients). Type of immunotherapy (PD-1 monotherapy vs. nivolumab plus ipilimumab), age and sex were not associated with the frequency of irAEs (Fine–Gray sub-distribution hazard model p = 0.71, p = 0.95, p = 0.13, respectively). More information can be seen in Figure S1.

Table 1. Baseline characteristics of the patients considering the diagnosis of immune-related adverse events during the study (*n* = 319).

Characteristics	*N*	Total	No irAEs (*n* = 150)	irAEs Grade 1–2 (*n* = 58)	irAEs Grade 3–4 (*n* = 111)	*p*-Value
Age at therapy start mean (±SD)	319	65.5 (±14.4)	65.9 (±14.7)	64.9 (±14.4)	65.3 (±14.0)	0.85 Anova
Sex;	319					
Female *n* (%)		127 (39.8)	65 (43.3)	21 (36.2)	41 (36.9)	0.48 Chi
Male *n* (%)		192 (60.2)	85 (56.7)	37 (63.8)	70 (63.1)	
Tumor localization;	319					
Head and neck *n* (%)		54 (16.9)	22 (14.7)	10 (17.2)	22 (19.8)	0.93 Fisher
Trunk *n* (%)		73 (22.9)	35 (23.3)	15 (24.1)	23 (21.6)	
Extremity *n* (%)		109 (34.2)	51 (34.0)	22 (36.2)	36 (33.3)	
Others *n* (%)		15 (4.7)	9 (6.0)	2 (3.4)	4 (3.6)	
Unknown *n* (%)		68 (21.3)	33 (22.0)	11 (18.9)	24 (21.6)	
Histological subtype;	319					
SSM *n* (%)		77 (24.1)	38 (25.3)	11 (18.9)	28 (25.2)	0.0005 Fisher
NM *n* (%)		73) (22.9)	30 (20.0)	14 (24.1)	29 (26.1)	
ALM *n* (%)		30 (9.4)	12 (8.0)	9 (13.8)	9 (9.0)	
LMM *n* (%)		13 (4.1)	4 (2.7)	5 (8.8)	4 (3.6)	
Uveal or ciliar body *n* (%)		13 (4.1)	0 (0)	9 (15.5)	4 (3.6)	
Others * *n* (%)		40 (12.5)	21 (14.0)	7 (12.1)	12 (10.8)	
Unknown *n* (%)		73 (22.9)	45 (30.0)	5 (6.9)	23 (21.6)	
BRAF status;	319					
WT *n* (%)		197 (61.8)	89 (59.3)	36 (62.1)	72 (64.9)	0.51 Chi
BRAFV600 *n* (%)		93 (29.2)	49 (32.7)	13 (22.4)	31 (27.9)	
Unknown *n* (%)		29 (9.1)	12 (8.0)	9 (15.5)	8 (7.2)	
Kit mutation;	319					
WT *n* (%)		158 (49.5)	64 (42.7)	28 (48.3)	66 (59.5)	0.84 Fisher
Yes *n* (%)		15 (4.7)	7 (4.7)	3 (5.1)	5 (4.5)	
Unknown *n* (%)		146 (45.8)	79 (52.7)	27 (46.6)	40 (36.0)	
LDH baseline;	299					
Normal *n* (%)		204 (68.2)	85 (65.5)	41 (70.7)	78 (70.3)	0.65 Chi
Elevated *n* (%)		95 (31.8)	45 (34.6)	17 (29.3)	33 (29.7)	
S100 baseline;	305					
Normal *n* (%)		170 (55.7)	70 (51.5)	33 (56.9)	67 (60.4)	0.37 Chi
Elevated *n* (%)		135 (44.3)	66 (48.5)	25 (43.1)	44 (39.6)	
Number of organs with metastases;	319					
1–3 *n* (%)		285 (89.3)	130 (86.7)	52 (89.7)	103 (92.8)	0.28 Chi
>3 *n* (%)		34 (10.7)	20 (13.3)	6 (10.3)	8 (7.2)	
Patients with brain metastases *n* (%)	319	61 (19.1)	41 (27.3)	11 (19.0)	9 (8.1)	<0.001 Chi
Patients with liver metastases *n* (%)	319	115 (36.1)	50 (33.3)	24 (41.4)	41 (36.9)	0.54 Chi
First-line immunotherapy	319					
PD-1 monotherapy *n* (%)		174 (54.6)	82 (54.7)	31 (53.4)	61 (54.9)	0.98 Chi
Nivolumab plus ipilimumab *n* (%)		145 (45.5)	68 (45.3)	27 (46.6)	50 (45.1)	
Best overall response	319					
Complete response *n* (%)		50 (15.7)	18 (12.0)	7 (12.1)	25 (22.5)	<0.001 LL
Partial response *n* (%)		80 (25.1)	22 (14.7)	19 (32.8)	39 (35.1)	
Stable disease *n* (%)		62 (19.4)	34 (22.7)	12 (20.7)	16 (14.4)	
Progressive disease *n* (%)		127 (39.8)	76 (50.7)	20 (34.5)	31 (27.9)	

WT: wild-type; PD-1 programmed cell death protein 1. irAEs: immune-related adverse events, Anova: one-way analysis of variance, Chi: Chi square-test, LL: linear-by-linear association test, Fisher: Fisher test. * Others: desmoplastic, polipoid, amelanotic melanoma. SSM: Superficial spreading melanoma, NM: Nodular melanoma, ALM: Acral lentiginous melanoma, LMM: Lentigo maligna melanoma.

The cumulative incidence of grade 1 or higher irAEs in the PD-1 monotherapy group at 1, 3, and 6 months was 23.8%, 39.7%, and 48.9%, and in the nivolumab plus ipilimumab group was 26.9%, 43.2%, and 52.3%, respectively.

3.2. Association between irAEs and Response

The DC rate was 60.2% for the whole collective, 69.8% for patients with any grade irAEs, and 49.3% for patients without irAEs (Table 1). The median duration of therapy in DC and PC groups was 7 months (IQR: 4–14) and 2 months (IQR: 1–3), respectively. Patients with DC had a significantly higher irAEs rate compared to those with PD. Considering the type of irAEs, all were strongly associated with response (Table 2).

Table 2. Response considering the presence and type of immune-related adverse events.

Type of Immune-Related Adverse Event	Disease Control (n = 192)	Progressive Disease (n = 127)	p
irAEs n (%)	118 (61.5)	51 (40.2)	<0.001
Hematological irAEs n (%)	117 (60.9)	46 (36.2)	<0.001
Hepatic irAEs n (%)	62 (32.3)	19 (14.9)	0.001
Renal irAEs n (%)	72 (37.5)	20 (15.7)	<0.001
Endocrine irAEs n (%)	60 (31.3)	17 (13.4)	<0.001

Patients with complete response (CR), partial response (PR), and stable disease (SD) as best overall response were considered to have disease control (DC), and patients with progressive disease (PD) were considered to have primary resistance, irAEs: immune-related adverse events.

Table 3 presents the binary logistic regression analysis of potential predictors of DC. After adjusting for confounding factors, the presence of any type of irAEs grade 3–4 was found to be associated with a protective effect for the outcome PD (adjusted OR (ORadj): 0.40, 95% CI 0.23–0.70, p = 0.001). This model was adjusted by age, sex, S100 values, number of metastases, and type of immunotherapy. The Hosmer–Lemeshow test showed goodness of fit of the model (HL: χ^2 = 8.374 p = 0.398 df = 8).

Table 3. Binary logistic regression analysis of protective factors in relation to the outcome progressive disease (n = 319).

Variable	Univariate Analysis			Multivariate Analysis		
	OR	95% CI	p-Value	OR	95% CI	p-Value
Age > 65 years	0.90	0.57–1.41	0.63			
Sex, male	0.63	0.40–0.99	0.05	0.74	0.45–1.23	0.24
S100 high	2.82	1.75–4.53	<0.001	2.51	1.53–4.15	<0.001
Brain metastases	1.16	0.66–2.03	0.62			
Liver metastases	1.42	0.89–2.26	0.14			
More than 3 metastases	1.82	0.89–3.71	0.10	1.59	0.71–3.60	0.26
Immunotherapy first-line						
Nivolumab	1			1		
Nivolumab/ipilimumab	0.74	0.47–1.16	0.19	0.63	0.38–1.04	0.07
Grade of irAEs						
No irAEs	1			1		
Grade 1–2	0.51	0.27–0.96		0.54	0.28–1.04	0.07
Grade 3–4	0.38	0.22–0.64		0.40	0.23–0.70	0.001

irAEs: immune-related adverse events.

3.3. Association between irAEs and Progression-Free Survival

In our cohort, the 3, 6, and 12 months PFS was 53.0%, 45.0%, and 38.7% respectively with a median PFS (mPFS) of 4 months (95% CI 3–7). We analyzed the association of PFS with irAEs stratified for low grade (CTCAE grade 1–2) and high grade (CTCAE grade 3–4). The mPFS in patients with no irAEs, low-grade irAEs, and high-grade irAEs was 3 months (95% CI, 2–3), 6 months (95% CI 3–16), and 15 months (95% CI 7-NA), respectively. At the 3-months landmark survival analysis, PFS was associated with the presence of irAEs (log-rank test = 0.05) (Figure 1). When PFS was analyzed considering the number of irAEs, patients with >5 irAEs (70 patients) and 1–5 irAEs (99 patients) had a statistically significant longer mPFS compared to patients with no irAEs (150 patients) (mPFS: 10 months [95% CI: 3–17], 9 months [95% CI: 0–18] and 3 months [95% CI: 2–3] $p < 0.01$, respectively).

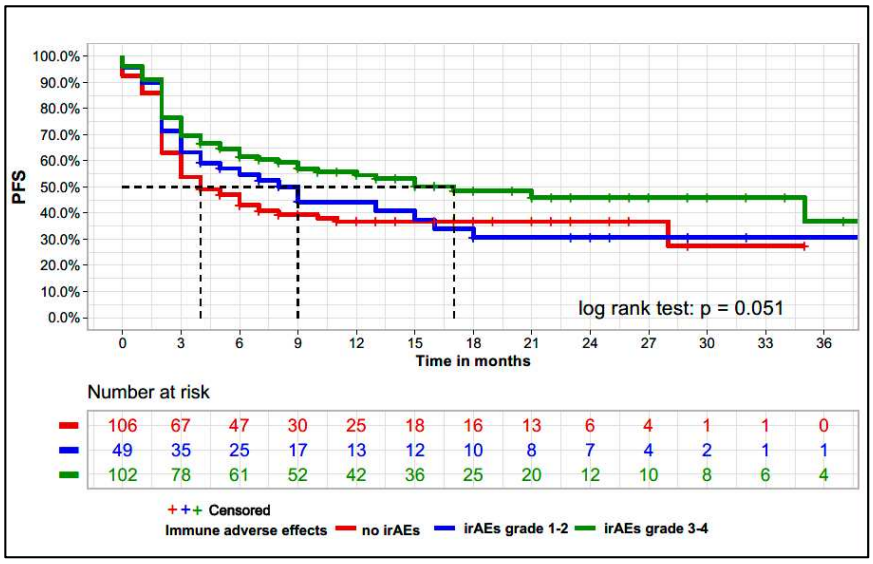

Figure 1. Kaplan–Meier curves for progression-free survival considering the presence of immune-related adverse events and respective CTCAE grade Kaplan–Meier curve for the 3-months landmark survival analysis of irAEs, which excluded patients who were lost to follow-up or died prior to this time of 3 months after starting immunotherapy (n = 257). irAEs: immune-related adverse events. Median PFS: not irAEs: 3 months (95% CI, 2–3), irAEs grade 1–2: 6 months (95% CI 3–16) and irAEs grade 3–4: 15 months (95% CI 7-NA).

PFS was also evaluated in the predetermined sub-types of irAEs (hematological, hepatic, endocrine, and renal). In all pre-specified sub-types, patients with grade 3–4 irAEs had better PFS outcomes than those with irAEs grade 1–2 or no irAEs (log-rank test: $p = 0.02$, $p = 0.005$ and $p = 0.006$ for hematological, renal, and endocrine irAEs, respectively), except in the subgroup with hepatic irAEs where patients with grade 3–4 irAEs had worse outcome ($p = 0.04$) (Figure S2). The 12 months PFS rate for patients without hepatic irAEs and those with hepatic irAEs CTCAE grade 1–2 and CTCAE grade 3–4 was 43.7%, 58.6%, and 29.2%, respectively.

A time-dependent Cox regression model was used to estimate the association between PFS and irAEs. In the univariate analysis, besides irAEs, S-100 levels and the presence of brain or liver metastasis were also associated with PFS). These variables were further integrated into a multivariate extended Cox regression model. The type of immunotherapy was also included, as this is a clinically relevant variable. The occurrence of irAEs grade 1–2 and 3–4 was not associated with a longer PFS; irAEs grade 1–2: HRadj 1.21 [95% CI: 0.91–1.79] $p = 0.16$ and irAEs grade 3–4 HRadj: 1.14 [95% CI: 0.83–2.02] $p = 0.24$ (Figure 2). On the contrary, immunotherapy with nivolumab plus ipilimumab was independently associated with longer PFS. An additional investigation included the interaction between the presence of irAEs and the type of immunotherapy received (PD-1 monotherapy vs.

nivolumab plus ipilimumab), which was not significant (p = 0.50). Different tests and graphical strategies were used to check the proportionality assumption of the Cox model. The Schoenfeld residual suggested evidence of proportionality (p global = 0.125). In addition, we performed a proportional cause-specific hazards model including irAEs as the time-dependent variable and mortality as the competing risk. The estimated sub-distribution hazard ratio [33] of the variable irAEs was also not significant (irAEs grade 1–2 HR: 1.32 95% CI: 0.94–1.85, p = 0.11, irAEs grade 3–4 HR: 1.16 95% CI 0.65–2.03 p = 0.62).

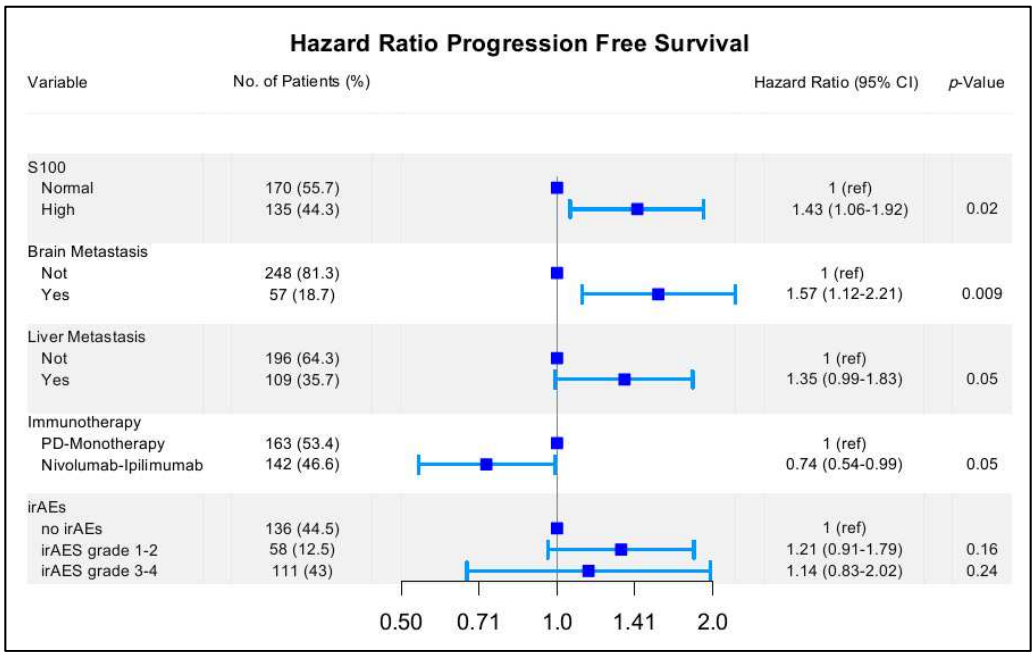

Figure 2. Multivariate analysis, time-dependent Cox regression model (progression-free survival) irAEs: immune-related adverse events.

3.4. Association between irAEs and Overall Survival

At the 3-months landmark survival analysis, the median OS (mOS) in patients with no irAEs, irAEs grade 1–2, and irAEs grade 3–4, was 21 months (95% CI 15-NA), 29 months (95% CI 20-NA) and not reached (95% CI, 29-not reached), respectively. OS was associated with the presence of irAEs (log-rank test < 0.001) (Figure 3). The 12-months OS in the groups with no irAE, irAEs grade 1–2, and irAEs grade 3–4 was 64.9%, 74.5%, and 86.5%, respectively. When OS was analyzed according to the number of irAEs developed, patients who developed ≥5 irAEs and 1–5 irAEs had a longer mOS compared to those without irAEs (mOS: NR [95% CI: 29-NR], NR [95% CI: 26-NR], and 21 months [95% CI: 15-NR] p < 0.001). Figure S3 displays the association between OS and the predefined sub-groups of irAEs. All sub-types of irAEs were significantly associated with improved OS.

Subsequently, we performed OS univariate analysis that included irAEs as a time-dependent variable. The univariate analysis underlined an association between OS and brain or liver metastasis, elevated S-100 values, and the presence of irAEs. The interaction between the type of immunotherapy received (PD-1 monotherapy vs. nivolumab plus ipilimumab) and irAEs were not statistically significant. Multivariable analysis confirmed that irAEs grade 1–2 (HRadj 0.61, 95% CI 0.40–0.93, p = 0.02) and irAEs grade 3–4 (HRadj 0.55, 95% CI 0.31–0.99, p = 0.04) were significantly associated with increased OS (Figure 4). The Schoenfeld residual suggested evidence of proportionality (p global = 0.118).

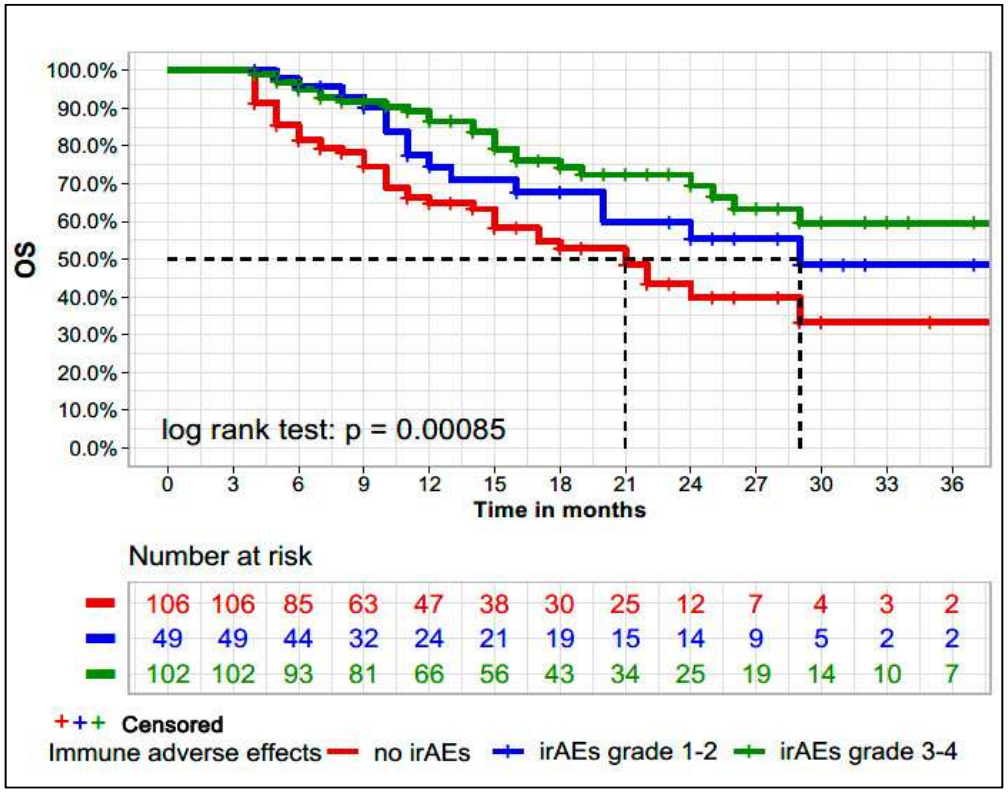

Figure 3. Kaplan-Meier plot for overall survival considering the presence of immune-related adverse events and respective CTCAE grade. Kaplan-Meier curve for the 3-months landmark survival analysis of irAEs, which excluded patients who were lost to follow-up or died prior to this time of 3 months after starting immunotherapy (n = 257). irAEs: immune-related adverse events. Median OS: not irAEs: 21 months (95% CI 15-NA), irAEs grade 1–2: 29 months (95% CI 20-NA), and irAEs grade 3–4: not reached (95% CI, 29-not reached).

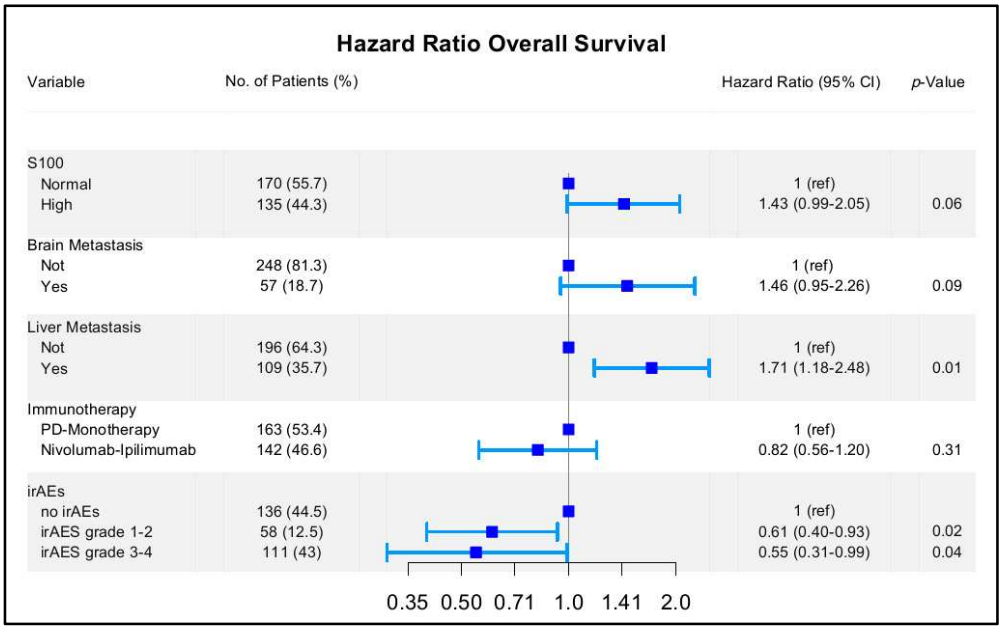

Figure 4. Multivariate analysis, time-dependent Cox regression model (overall survival) irAEs: immune-related adverse events.

4. Discussion

In the present study, we found that the development of irAEs, as expressed by changes in laboratory values, is significantly associated with disease control in patients with stage IV melanoma treated with PD-1-based immunotherapy indicating that irAEs can be a predictive factor for ICI. The statistically significant association between irAEs and DC was seen in all the pre-defined sub-types of irAEs. The presence of irAEs was also significantly associated with an improved OS and a trend was seen for PFS. Finally, we confirmed the prognostic value of other known factors in stage IV melanoma, such as the presence of elevated S100 levels and the presence of liver and brain metastases.

In our cohort, the rate of irAEs of any grade was 53%, similar to other reports using daily routine data [1,13], but lower than the rates previously reported in clinical trials [34–36]. Hematologic and renal immune-related adverse events were reported more frequently in this work than in other comparable studies. The reason for this is probably that we performed a systematic evaluation of all laboratory findings for each patient and found relatively frequent findings of white and red blood cells deviating from the normal value and deviating values for creatinine.

The time to onset of irAEs described in other publications varies between 2 and 16 weeks [18,19,34,37]. In our cohort, the median time to onset of irAEs was 12 weeks (95% CI 12–20), which is longer than previously reported, probably because we did not consider the irAEs that have an earlier onset, such as cutaneous and gastro-intestinal irAEs, and included those with later onset as endocrine and renal irAEs [19,38]. The time of onset of irAEs, however, does not seem to be associated with response, as publications involving different tumor entities, including in melanoma, show conflicting results [21].

The association between irAEs and response to ICI has been concordantly described in melanoma, renal cell carcinoma, and non-small cell lung cancer patients [1,34,39–43]. In a pooled analysis of four trials including 576 patients with advanced melanoma treated with nivolumab, the presence of irAEs was significantly associated with ORR [34]. The ORR in patients with any irAEs was higher compared to those patients with no irAEs (ORR 48.6% vs. 17.8%; 95%CI: 42.3–54.9 and 13.7–22.4). In our cohort, the DC rate in patients with any grade irAEs was 69.8% and 49.3% in those without irAEs. The difference might be related to the sub-type of irAEs reported by Weber et al. and in our cohort, and to the type of treatment received. Weber et al. reported the ORR for patients treated with nivolumab monotherapy, 54% of which had received prior ipilimumab therapy, while in our cohort we only included patients receiving first-line immunotherapy, 45.5% of which received nivolumab plus ipilimumab [34]. The question arises, however, as to whether patients with disease progression had even spent enough time on treatment to develop immune-mediated adverse events. Of the 127 patients with progressive disease, 94 had already died during the observation period; the median survival time of this collective was 7 months. The median progression occurred after 2 months and the median duration of treatment was also 2 months. It is, therefore, possible that this short duration of treatment contributed to fewer immune-mediated adverse events being observed.

The benefit in terms of PFS for patients with irAEs seems to be quite consistent in non-small cell lung cancer, and gastro-intestinal malignancies [41,43–45]. As for melanoma, in the pooled analysis reported by Weber et al., there seems to be no benefit in terms of PFS for patients with irAEs [34]. In our analysis, a trend in terms of PFS benefit was seen when comparing patients without irAEs and with grade 1–2 or 3–4 irAEs ($p = 0.051$), but this trend was not confirmed in the multivariate time-dependent Cox analysis. However, Indini et al. reported that the presence of irAEs was the only factor independently associated with improved PFS [1]. The differences in terms of populations included, the sub-type of irAEs reported, the fact that we analyzed the irAEs in two groups (grade 1–2 and grade 3–4) instead of all together, and the different systemic therapies might justify these differences. In addition, PFS was very short for patients with tumor progression especially in those patients with primary resistance, the median PFS was only 2 months. For the total group of

patients with onset of tumor progression, the median PFS is 4 months. This short period of time may not be enough to reveal the effects of an immune response.

Contrary to the PFS benefit, the benefit in terms of OS for patients with irAEs seems to be more homogenous, aligned with our current report, where the mOS in the groups with no irAE, irAEs grade 1–2, and grade 3–4 was 21 months, 29 months and not reached, respectively ($p < 0.001$). The presence of irAEs was also independently associated with OS in the multivariate time-dependent Cox regression analysis. Indini et al. also reported a benefit in OS, particularly for patients with vitiligo, compared to those without irAEs (median OS 9.7 months for no irAEs vs. 21.9 months for other irAEs vs. not reached for patients with vitiligo) [1]. In another publication that included data from 148 patients with melanoma treated with nivolumab, a statistically significant OS difference was noted among patients with irAE compared to those without irAEs ($p < 0.001$) [13]. Finally, in the adjuvant setting, particularly in high-risk stage III melanoma, a statistically significant association between irAEs and improved recurrence-free survival and OS was also reported [8].

In our cohort, the association between irAEs and improved OS was present in all predefined categories of irAEs, except in patients with hepatic irAEs. In this subgroup, we saw that grade 1–2 hepatic irAEs but not grade 3–4 irAEs were associated with favorable outcomes. When analyzing the proportion of patients with irAEs grouped by presence or absence of liver metastases, 42.6% of patients with liver metastases had hepatic irAEs compared to 29.9% of patients without liver metastases. For the survival analysis of patients with hepatic irAE, we excluded patients with liver metastases and elevated liver enzymes at baseline. Nevertheless, we cannot completely exclude that the worse outcome seen is due to the presence of unrecognized liver micrometastases and/or later hepatic progressive disease, which can translate into liver enzymes elevation.

Immune modulation resulting from ICIs can alter normal self-tolerance, which clinically translates into irAEs. Despite intense research on the topic, the exact mechanisms by which irAEs are triggered are still not clear [46,47]. Studies suggest that irAEs could be caused by antigens that are common to both tumor and affected organ, leading to a cytotoxic effect on normal cells. Treatment with ICIs increases T-cell activation and proliferation leading to increased production of proinflammatory cytokines triggering a nonspecific activation of the immune response, with non-specific inflammation and autoimmunity [37,48,49]. In addition, anti-PD-1 therapy may also affect humoral immunity, leading to increased levels of pre-existing autoantibodies [50,51]. The combination of these mechanisms would lead to hyperactivation of the immune system, translating into a higher rate of irAEs but also into a better tumor response. The challenge here is to uncouple tumor response and toxicity. Available data are scarce, and it is still unclear if this is at all possible [8]. In our cohort, premature treatment discontinuations were rare (~5%) and we generally observed stable remission in these patients.

Strengths and Limitations: Our study has several strengths. First, the reliability of the data used and the severity grade attributed to each irAEs were not dependent on clinical documentation as it is, for example, for cutaneous, rheumatological, gastro-intestinal, or lung toxicities. Here, we used an established classification for irAEs (CTCAE) which allows for future comparisons with other analyses. Second, the study includes many patients with an adequate median follow-up time, which allowed a precise estimation of the association between irAEs and the outcomes DC, PFS, and OS, as well as subgroup analyses. Third, we used adequate statistical methods to avoid bias, and we adjusted the analyses for possible confounders.

Regarding limitations of this study, it is important to point that this is a retrospective study, thus bias in patients' selection can be present. Moreover, due to its retrospective nature and the strategy used for irAE identification, not all irAEs were included, and this could have affected our analysis.

5. Conclusions

The presence of irAEs was positively and significantly associated with DC and OS. This observation was stable in all the Cox regression models performed. Our data show that the presence of irAEs may predict DC in patients with advanced melanoma receiving ICI. However, to adequately compare and investigate the predictive effect of irAEs across tumor entities and irAEs sub-types, a standardized collection of irAEs is necessary.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13236141/s1, Figure S1: Number of immune-related adverse events by time-period and cumulative incidence of immune-related adverse events, Figure S2: Kaplan–Meier curves for progression-free survival by type of immune-related adverse events, Figure S3: Kaplan–Meier curves for overall survival by type of immune-related adverse events.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to data protection.

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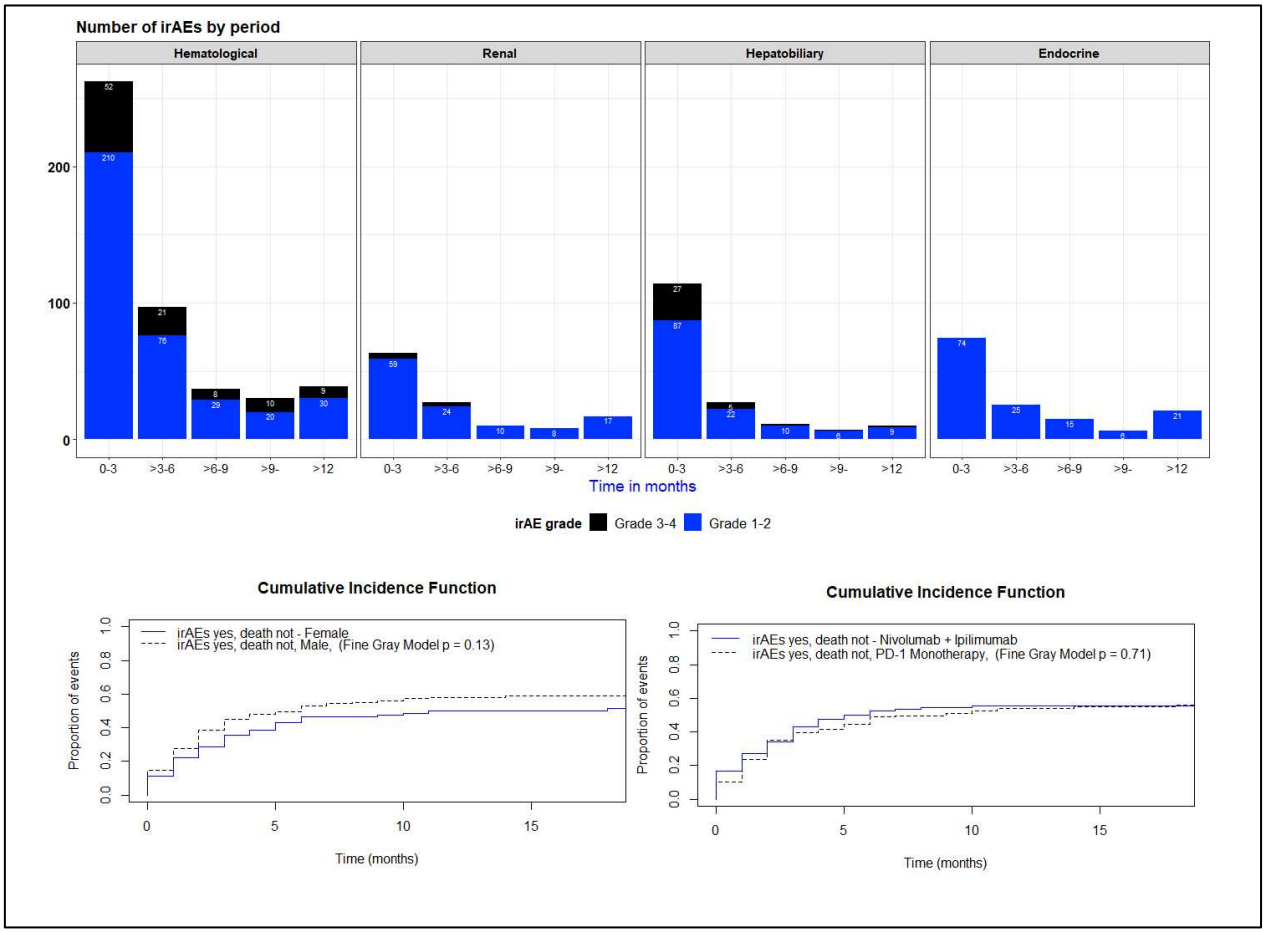
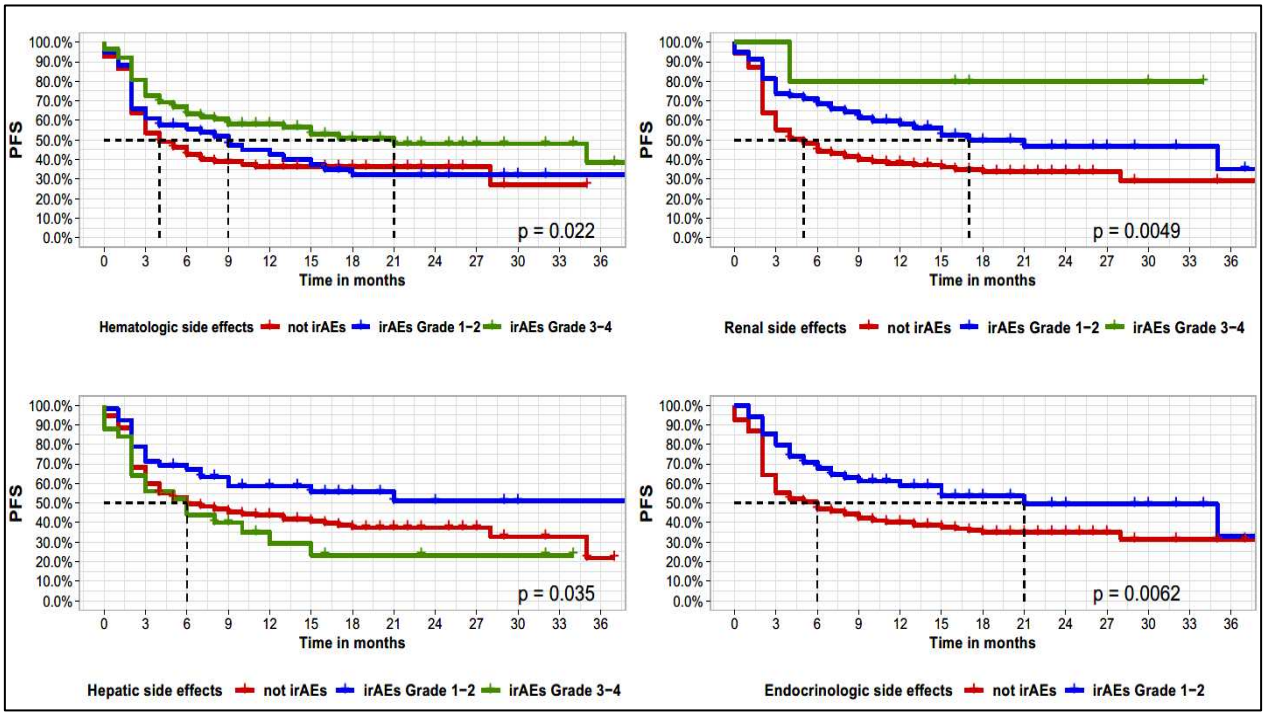
SUPPLEMENTS

Supplementary Figure 1: Number of immune related adverse events by time-period and cumulative incidence of immune related adverse events



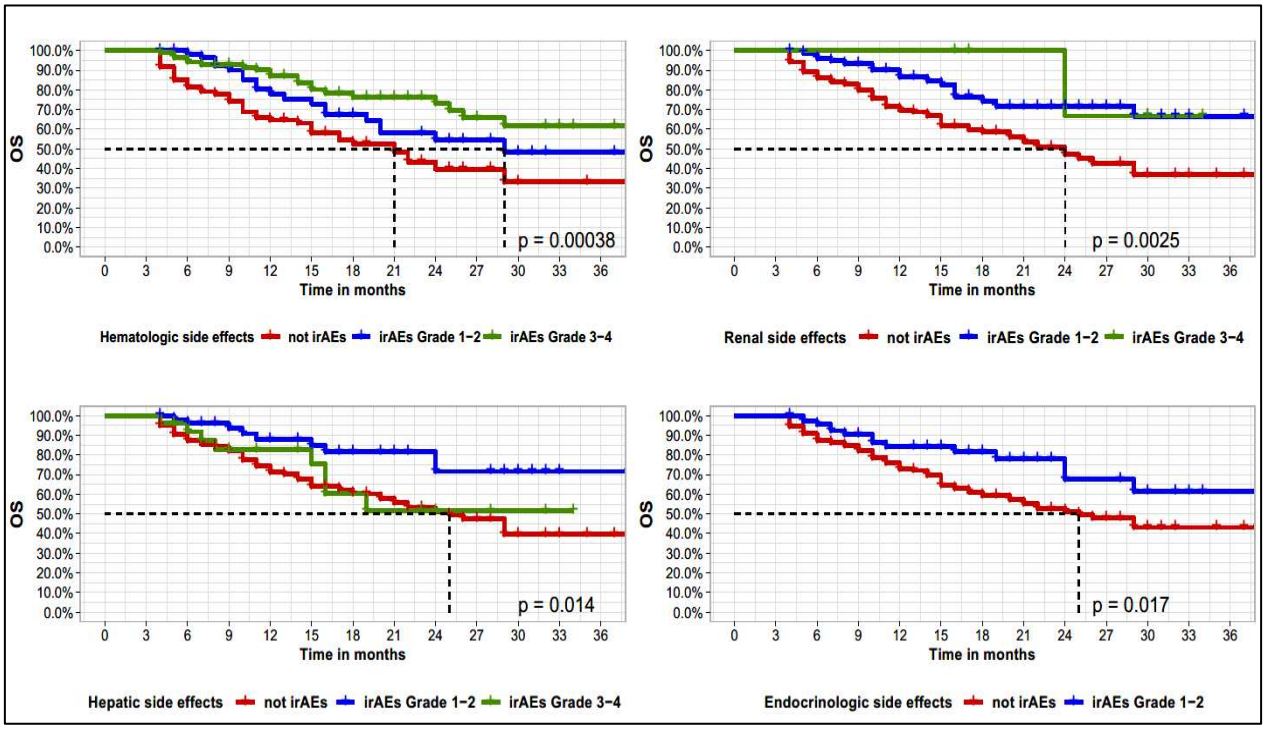
n= number of patients at therapy by period, *cumulative incidences adjusting for competing risks,
Note: 1 patient can have more than one side effect per period.

Supplementary figure 2: Kaplan Meier curves for progression free survival by type of immune related adverse events, excluding patients who were lost to follow-up or died in the first 3 months after starting immunotherapy (n=257) (Landmark survival analysis).



irAEs: immune-related adverse events

Supplementary figure 3: Kaplan Meier curves for overall survival by type of immune related adverse events, excluding patients who were lost to follow-up or died in the first 3 months after starting immunotherapy (n=257) (Landmark survival analysis).



irAEs: immune-related adverse events

4 DISCUSSION

4.1 Most important findings

4.1.1 First study: **"Modifiable Risk-factors for Keratinocyte Cancers in Australia: A Case-control Study (1)"**

The first study included data collected in North Queensland as a part of the Non-Melanoma Skin Cancer Project (Australia). We assessed the demographic characteristics, lifestyle-related sunlight exposure habits, sun protective practices, and other potential KC risk factors. We found an increased risk of KC associated with the duration of daily sun exposure, distribution and extent of freckling on the face, forearms, and shoulders during adolescence, lower academic qualification, solar lentigines on the shoulders, and propensity to sunburn. The results of this study highlight the role of the individuals' phenotypic characteristics and sunlight exposure as significant risk factors for the development of KC (26–28).

Solar UV radiation is the most important environmental risk factor for developing skin cancer (26). Approximately 90% of KCs are attributed to UV radiation (29). UV radiation induces DNA damage by either the direct excitation of DNA or by the indirect excitation of other endogenous non-DNA chromophores (26). Both contributed to generating reactive oxygen species (ROS), transition mutations, cytokine-mediated inflammation, and ultraviolet-mediated immunosuppression (27). All these can eventually lead to KC (26).

Concerning the amount of time spent in the sun during different age periods, we found that high sun exposure in adulthood is a risk factor for KC, regardless of exposure during childhood. This finding persisted after adjustment for other variables known to affect KC risk. Our results are similar to previous studies, including a cohort study of 56,667 women, where adulthood UV radiation exposure increased the risk of KC regardless of childhood UV radiation exposure (28). Nevertheless, this is in contrast to other studies that highlighted early-life sunlight exposure as one of the most significant predictors of adult skin cancer

(30) and consider young childhood and adolescence a period when individuals have greater vulnerability to toxic exposures, including UV radiation (30). Iannacone et al. conducted a case-control study (218 BCC, 169 SCC, and 316 controls) investigating sun-exposure timing as a risk factor for BCC and SCC. In this study, exposure to sunlight during early years was associated with an increased risk for SCC but not for BCC (24). Based on these findings, it seems necessary to clarify whether solar UV exposure is more harmful during vulnerable periods in life.

As solar UV radiation is the most important environmental risk factor for developing skin cancer (9), several approaches have been established to reduce UV radiation exposure. These practices include avoiding direct exposure to midday sun (between 10 am and 2 pm), textile protection with appropriate clothing, and sunscreen with a minimum sun protection factor of 15-20 (26, 31). Interestingly, in our study, frequent use of sunscreen had no protective effect on the risk for KC. This finding is consistent with other studies, such as Green et al., who did not find significant differences in the incidence rate of KC between groups randomly assigned to daily sunscreen or no daily sunscreen use (BCC RR 1,03, 95% IC 0.73-1.46; and SCC RR 1.35 (95% IC 0.84-2.19)) (32). On the contrary, Van der Pols et al. evaluated the effect of regular sunscreen application on the future development of BCCs and SCCs in 1,484 participants. After eight years of follow-up, they found a significantly reduced incidence rate of SCC (RR 0.62 95% IC 0.38-0.99), whereas a no-significant decrease was shown for BCC (RR 0.75 95% IC 0.49-1.14). However, the authors also reported that the amount of time spent outdoors on weekdays and weekends was similar between both treatment groups (34). At the same time, in our study, the duration of daily sun exposure was higher in cases than in controls. This might explain our lack of correlation found regarding the use of sunscreen.

Interestingly, in our study, the use of sunscreen did not significantly correlate with the development of KC, which could be explained by a false sense of protection while using sunscreen, encouraging sunlight exposures of longer durations, ultimately leading to a delay of sunburn occurrences instead of their prevention (31–33). Furthermore, the effectiveness of sunscreen use largely depends on the amount applied, application frequency, the sun protection factor used, and the

individual's skin phototype (33,35–37). Other recommended strategies to prevent skin cancer would be to avoid excessive sunlight exposure by seeking shade, reducing the time of exposure by minimizing outdoor activity during periods of peak ambient UV radiation (31,35), textile protection (26,33), and regular self-examination or clinical examination, allowing early detection of any skin changes (26).

Our study found that many cases and controls engaged in numerous skin cancer risks behaviors, such as infrequent sunscreen use, shirt and hat use, and duration of daily sun exposure of more than 4 hours. However, when these variables were compared between different time intervals, trends in sun protection behaviors (wearing a hat, shirt, and sunscreen) increased significantly from 1970-2010. This finding probably resulted from mass media campaigns in Australia, which were introduced in 1980 to raise public awareness about skin cancer, the risks of exposure to UV radiation, and the need for sun protection (38,39). This finding highlights the importance of ongoing and future skin health campaigns encouraging the population to use preventive measurements and participate in regular skin cancer screening programs (25,39).

Since 1985, several studies with various designs have reported that smokers have an elevated risk for a subsequent SCC (15,17,41,42) but not in BCC (18). The effect of smoking on the development of SCC has been attributed to several classes of compounds with demonstrated carcinogenic activity within cigarette smoke and a suppressive effect on immunologic functions (15). Our results, however, do not show an association between smoking and the development of KC and are instead in agreement with another Australian study, where current smoking was not a risk factor for SCC after adjusting for other known risk factors (Relative risk RR 1.1 95% IC 0.7-1.5). The lack of a link between smoking and SCC in Australia might be explained by the extremely high levels of ambient solar UV exposure, potentially overriding a weak causal effect of smoking if it exists (43).

Although several studies have examined a possible connection between alcohol intake or the type of alcohol consumed and the development of KC, no definitive links were identified (18,44–47). There are several lines of evidence showing that alcohol metabolites may increase the risk of skin cancer (48), including the

increase in oxidative stress leading to DNA damage (49), as well as interference with immune functions, which might increase the propensity to develop cancer (44). However, our data provide only a non-significant trend between the risk for KC and the liquor intake amount. Hence, further investigations are necessary to evaluate a causal association between alcohol consumption and the risk for KC.

KCs are known to be associated with states of immune perturbation (27,50). Studies in patients with rheumatoid arthritis, who received an immune-modulatory biologic drug had a 20-80% increased risk of KC than the general population (52). In contrast, our results showed no significant differences between cases and control concerning the use of immunosuppressive therapy

The present study has several limitations.

First: there is a lack of data concerning the quantity of sun exposure (i.e., at midday vs. mornings or late afternoons). Second: the survey was self-reported, and recall bias is possible because individuals with KC are more concerned about possible causes of their disease and are thus more likely to remember their exposure history than controls.

A strength of this study is the availability of data on many potential risk factors, including sun exposure and sun protection strategies in different time intervals, allowing adjustment of confounding factors. Another strength is that controls were screened for current signs of BCC and SCC by a medical expert to avoid misclassification of the case-control status that might result from self-reported data.

Conclusion:

Our findings primarily support the increased risk of KC in association with sun exposure, and these findings are consistent with those previously reported in other studies. Importantly, we showed that the frequency of sun-protective practices did not differ statistically significantly between cases and controls. Further investigations are needed focusing on these variables, together with individual susceptibility factors and other potential interacting factors leading to

an increased risk for KC development, to define which preventive strategies are most effective to reduce the risk for KC

4.1.2. Second study: **"Association between Immune-Related Adverse Events and Survival in 319 Stage IV Study Melanoma Patients Treated with PD-1-Based Immunotherapy: An Approach Based on Clinical Chemistry (100) "**

In this real-world study, the development of irAEs was associated with a better OS and BOR rate in patients with unresectable stage IV melanoma treated with PD-1-based immunotherapy, indicating that treatment- related irAEs might represent a predictor of ICIs efficacy in melanoma patients (100).

In the last decade, ICIs have changed survival outcomes for patients with advanced cancers (102, 119, 122, 169). Anti-PD-1 antibodies target the programmed death receptor ligand-1 (PD-L1), expressed by various tumors and antigen-presenting cells in the tumor microenvironment (120). The binding of PD-1 to its ligands inhibits T-cell proliferation and the production of proinflammatory cytokines (109, 111, 119). Consequently, anti-PD-1 antibodies prevent this binding, producing an enhanced anti-tumor immune-mediated response (108). Several studies have demonstrated improved OS and PFS in randomized phase III trials with anti-PD-1 antibodies (pembrolizumab and nivolumab) (119, 170), being the first choice immunotherapy for advanced melanoma treatment, which can be optionally combined with ipilimumab (111).

However, therapy with ICIs is often associated with irAEs (102, 113, 116, 171, 172). They usually occur within a median onset period ranging from 2 to 16 weeks (107, 124, 173). irAEs have been described with varying degrees of severity in different organ systems, ranging from mild inflammation to life-threatening organ damage (122). The most affected organs are the gastrointestinal tract, liver, skin and endocrine systems (116), and the most frequent irAE are rash, vitiligo, colitis, pneumonitis, hepatitis, thyroiditis, nephritis, and hypophysitis (117, 119). Anti-PD1 antibodies have a safer toxicity profile, with an overall lower incidence of

irAEs when compared to anti-CTLA-4 (119); and the combinations of these two drugs increases the severity and frequency of irAE up to 60% (101, 116).

The mechanisms leading to the development of irAEs are unclear. Some studies suggest that irAEs may be caused by antigens present in both tumor and inflamed organs, leading to a cytotoxic effect on healthy cells (128, 174). ICIs increase T-cell activation and proliferation, leading to an increased cytokine production that triggers a nonspecific immune response activation (67, 68, 209). In addition, anti-PD-1 therapy may also affect humoral immunity, leading to increased levels of pre-existing autoantibodies (175).

Retrospective studies have suggested that the development of irAEs is associated with a better therapeutic response to cancer than those who do not develop irAEs (107, 124, 176, 177), these findings indicate a potential link between autoimmunity and the antitumor effect observed by ICIs (107, 178). IrAEs appear to represent a clinical biomarker for ICIs response (128), but the mechanisms underlying this association are still not completely understood. It has been proposed that a close link exists between autoimmunity and the antitumor effect elicited by ICIs (128). ICIs could play a role in the non-maintenance of tolerance to self-antigens in humans and in tumor regression (118, 176).

There is increasing evidence that patients who have irAEs have marked improvements in PFS, OS, and BOR rates to those who did not develop an irAE, with more consistent data in patients treated with anti-PD-1 antibodies (107, 179) than in those treated with CTLA-4 inhibitors (180). In a retrospective analysis of 173 patients with metastatic melanoma treated with anti-PD-1 antibody, 59% experienced irAEs; in the multivariate analysis, the presence of irAEs was independently associated with PFS (HR 0.47; 95% CI 0.26-0.86; p=0.016) and OS (HR 0.39; 95% CI 0.18-0.81; p<0.001) (128). Freeman-Keller et al. performed a retrospective study on 148 melanoma patients treated with nivolumab. They found a statistically significant difference in OS in patients with irAEs compared to patients without irAEs (p<0.001) (119). A systematic review of 27 studies reported an association between the incidence of vitiligo with PFS (HR: 0.51) and OS (HR: 0.25) (181). Another systematic literature review of 36 studies concluded

that despite the revised studies' high heterogeneity and enormous bias, irAEs could be associated with the therapeutic activity of ICIs (182).

Our data also support the association between irAEs and better BOR and OS rates. Patients experiencing irAEs had a higher percentage of disease control compared to those who did not have irAEs (69.8% vs. 49.3%). In addition, the occurrence of grade 3 and 4 irAEs was significantly associated with a protective effect for the outcome primary resistance (OR: 0.40 95% CI 0.23-0.70, p=0.001). Lastly, the presence of any grade irAEs was significantly associated with longer OS (irAEs grade 1-2 HRadj: 0.61 95% CI: 0.4-0.93, p=0.02, irAEs grade 3-4 HRadj: 0.55 95% CI 0.31-0.99, p=0.04)

Interestingly, our analysis showed that grade 1-2 hepatological irAEs but not grade 3-4 irAEs were associated with favorable prognosis. However, analyzing the proportion of patients with irAEs grouped by a diagnosis of liver metastases, 42.6% of patients presented hepatological irAEs compared to 29.9% of patients without liver metastases, which could explain our findings.

Strengths and Limitations

Our study has several strengths. First, the study had a large sample and adequate median follow-up time, which allowed an accurate estimation of the association between irAEs and the outcomes BOR, OS and PFS, and subgroup analyses (113). Second, we used appropriate statistical methods to avoid bias and adjusted the analyses for potential confounders, including metastasis and therapy (100). Third, we performed a landmark analysis, which helps estimate the association between adverse events and treatment efficacy, as sometimes patients with more extended treatment periods are at a greater risk of developing adverse events (100, 183).

This study has several limitations. First, this was a retrospective study. Thus bias in patient selection can be present. Second, due to its retrospective nature and the strategy used for irAEs identification (by laboratory values), not all irAEs might have been recognized, especially those only clinically diagnosed, and any

unrecorded irAEs could have potentially influenced our analysis (171). Third, the definition used of irAEs (Common Terminology Criteria for Adverse Events) can underestimate or overestimate the severity of irAEs and can be challenging to apply in some organ-specific irAEs (for example, dermatological irAEs) (107, 117).

Conclusion: In this study, we observed a significant association between the development of irAEs with tumor response and overall survival. This observation remained stable all Cox regression models performed. The presence of irAEs may have the potential to be an effective surrogate and predictive marker of BOR and survival in ICIs therapy.

5 SUMMARY

First study:

Keratinocyte cancer (KC) is the most prevalent malignancy in Caucasians, with incidence having continued to rise over the past 40 years. Australia has the highest reported incidence of KC, with the most extreme incidence rates recorded in North Queensland. This study aims to investigate and elucidate the environmental and host risk factors responsible for keratinocyte cancer development in the high-risk population of Australia.

In this case-control study, cases were immune-competent adults from Townsville, Australia, who had a new basal cell carcinoma or squamous cell carcinoma histologically confirmed during 2004-2009. Cases were age-matched (±5years) to immune-competent, community-based controls from Townsville with no prior history of keratinocyte cancer

This study included 112 squamous cell carcinoma (SCC), 95 basal cell carcinoma (BCC), and 122 controls. Compared to the controls, both BCC and SCC were significantly less educated; they were more likely to have light eyes and light color hair, more freckling on the face, a higher amount of solar lentigines, and a greater tendency to burn from sunlight exposure. In the multivariate analysis, we found a significant association between SCC and lower academic qualification (OR=2.35 p=0.10), freckling (OR=1.04 p<0.01), solar lentigines (OR=1.02 p=0.01), the propensity to sunburn (OR=2.75 p=0.01) and a high number of accumulated hours of sunlight exposure (OR=2.43 p=0.04)**.** Additionally, we found a significant association between BCC and less propensity to sunburn (OR=2.68 p=0.01), freckling (OR=1.05 p<0.01), and a high or medium number of accumulated hours of sunlight exposure (high: OR=2.36 p=0.04; medium: OR=2.33 p=0.03). No significant differences were found with respect to gender,

history of internal cancers, smoking, alcohol consumption, diet, or use of sun protection (wearing a hat, long-sleeved shirt, and sunscreen).

This study provides evidence that a sun-sensitive phenotype and prolonged sun exposure contribute to the risk of developing KC. Notably, the frequency of sun-protective practices did not appear to reduce the risk of KC.

Second study

The prognosis for patients with advanced melanoma has improved significantly with the introduction of monoclonal antibodies targeting immune regulatory checkpoint receptors, such as anti-programmed cell death 1 (PD-1) and anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (101, 102), improving overall survival (OS) and reducing the risk of recurrence (101, 103, 113, 184, 185).

Therapy with immune checkpoint inhibitors (ICIs) is often associated with immune-related adverse events (irAEs) (113, 116, 186). They have been described with varying degrees of severity in different organ systems, ranging from mild inflammation to life-threatening organ damage (122). Recently published data has shown an association between irAEs and improved outcomes of patients treated with immune checkpoints inhibitor (101, 107, 113, 119, 122, 127). Given the immune mechanism of action of ICI, it is reasonable to associate the development of autoimmune events with improved outcome, as activation of the immune system could lead to both tumor response and autoimmunity (127). Reports of patients developing irAEs during treatment with ICIs have been contradictory regarding the impact of toxicity on survival outcomes, and a clear association between these two variables has not been found yet. In addition, it remains uncertain whether these observations can be explained by the role of irAEs as an indicator of drug activity (113). Finally, little is known regarding the impact of other variables on the association between irAEs and outcomes (113).

The primary aim of this cohort study was to assess the association between irAEs and disease control rate in patients with stage IV melanoma receiving first-line PD-1- based immunotherapy. Patients with complete response, partial response, and stable disease as the best overall response (BOR) according to RECIST 1.1 were included in the disease control group. Patients with progressive disease as the BOR were included in the primary resistance group. Secondary endpoints were PFS and OS.

Among 319 patients, 53% experienced at least one irAE. Patients who experienced irAEs had a higher percentage of disease control in comparison to those who did not, independently of the CTCAE grade (69.8% vs. 49.3%). In multivariate analysis, development of grade 3 and 4 irAEs was significantly associated with a protective effect for the outcome primary resistance (OR: 0.40 95% CI 0.23-0.70, p=0.001). The presence of any grade irAEs was significantly associated with increased OS (irAEs grade 1-2 HRadj: 0.61 95% CI: 0.4-0.93, p=0.02, irAEs grade 3-4 HRadj: 0.55 95% CI 0.31-0.99, p=0.04), but not with PFS (irAEs grade 1-2 HRadj: 1.21 95% CI: 0.91-1.79, p=0.16, irAEs grade 3-4 HRadj: 1.14 95% CI 0.83-2.02, p=0.24).

Conclusion: The occurrence of irAEs with laboratory expression is associated with a favorable response and OS, suggesting that irAEs may be a predictive factor in this setting.

6 DEUTSCHE ZUSAMMENFASSUNG

Erste Studie:

Keratinozytenkrebs (KC) ist die häufigste bösartige Erkrankung bei Kaukasiern, wobei die Inzidenz in den letzten 40 Jahren weiter zugenommen hat. Australien weist die höchste gemeldete Inzidenz von KC auf, wobei die extremsten Inzidenzraten in Nord-Queensland verzeichnet werden. Ziel dieser Studie war es, Risikofaktoren zu untersuchen, die für die Entwicklung von Keratinozytenkrebs in der australischen Hochrisikopopulation verantwortlich sein könnten.

Im Rahmen einer Fall-Kontroll-Studie wurden immunkompetente Erwachsene aus Townsville, Australien analysiert, bei denen zwischen 2004 und 2009 ein neues Basalzellkarzinom oder Plattenepithelkarzinom histologisch bestätigt wurde. Die Fälle wurden altersmäßig (±5 Jahre) mit immunkompetenten, gemeindebasierten Kontrollen aus Townsville ohne Vorgeschichte von Keratinozytenkrebs abgeglichen.

Diese Studie umfasste 112 Plattenepithelkarzinome (SCC), 95 Basalzellkarzinome (BCC) und 122 Kontrollen. Im Vergleich zu den Kontrollen waren sowohl BCC als auch SCC signifikant weniger gebildet; sie hatten eher helle Augen und helles Haar, mehr Sommersprossen im Gesicht, ein größeres Ausmaß an Lentigines solares sowie eine größere Neigung zu Sonnenbrand. In einer multivariaten Analyse wurde ein signifikanter Zusammenhang zwischen SCC und einer niedrigeren akademischen Qualifikation (OR=2,35 p=0,10), Sommersprossen (OR=1,04 p<0,01), Lentigines solares (OR=1,02 p=0,01), der Neigung zu Sonnenbrand (OR=2,75 p=0,01) und einer hohen Anzahl kumulierter Sonnenstunden (OR=2,43 p=0,04) festgestellt. Darüber hinaus wurde ein signifikanter Zusammenhang zwischen BCC und einer geringeren Neigung zu Sonnenbrand (OR=2,68 p=0,01), Sommersprossen (OR=1,05 p<0,01) und einer

hohen oder mittleren Anzahl an kumulierten Sonnenstunden festgestellt (hoch: OR=2,36 p=0,04; mittel: OR=2,33 p=0,03). Es wurden keine signifikanten Unterschiede in Bezug auf das Geschlecht, die Vorgeschichte Krebserkrankungen, das Rauchverhalten, den Alkoholkonsum, die Ernährung oder die Verwendung von Sonnenschutzmitteln (Hut, langärmeliges Hemd und Sonnenschutzmittel) festgestellt.

Diese Studie liefert weitere Hinweise darauf, dass ein sonnenempfindlicher Phänotyp und übermäßige Sonnenexposition zum Risiko der Entwicklung von KC beitragen. Bemerkenswert ist, dass die Häufigkeit der Sonnenschutzmaßnahmen das KC-Risiko nicht zu verringern scheint.

Zweite Studie

Monoklonale Antikörper, die auf die immunregulatorischen Checkpoint-Rezeptoren Anti-Programmed Cell Death 1 (PD-1) und Anti-Cytotoxic T-Lymphozyte-Associated Protein 4 (CTLA-4) abzielen, haben die Prognose von Patienten mit fortgeschrittenem Melanom erheblich verbessert [112, 113], das Gesamtüberleben (OS) verbessert und das Rezidivrisiko verringert [112, 114, 124, 177, 178].

Die Therapie mit Immun-Checkpoint-Inhibitoren (ICIs) ist häufig mit immunbezogenen unerwünschten Ereignissen (irAEs) verbunden [124, 127]. Sie wurden mit unterschiedlichem Schweregrad in verschiedenen Organsystemen beschrieben und reichen von leichten Entzündungen bis hin zu lebensbedrohlichen Organschäden [133]. Kürzlich veröffentlichte Daten zeigten einen Zusammenhang zwischen irAEs und verbesserten Behandlungsergebnissen bei Patienten, die mit Immun-Checkpoint-Inhibitoren behandelt wurden [112, 124, 130, 133, 138]. In Anbetracht des immunologischen Wirkmechanismus von ICI ist es naheliegend, die Entwicklung von Autoimmunereignissen mit einem verbesserten Ergebnis in Verbindung zu bringen, da die Aktivierung des Immunsystems sowohl zu einer Tumorreaktion

als auch zu Autoimmunität führen könnte [138]. Die Berichte über Patienten, die während der Behandlung mit ICIs irAEs entwickeln, sind widersprüchlich, was die Auswirkungen der Toxizität auf das Überlebensergebnis angeht, und ein eindeutiger Zusammenhang zwischen diesen beiden Variablen wurde bisher nicht gefunden. Darüber hinaus bleibt ungewiss, ob diese Beobachtungen durch die Rolle von irAEs als Indikator für die Arzneimittelaktivität erklärt werden können (74). Schließlich ist wenig über den Einfluss anderer Variablen auf den Zusammenhang zwischen irAEs und Behandlungsergebnissen bekannt [124].

Das primäre Ziel dieser Kohortenstudie war die Bewertung des Zusammenhangs zwischen irAEs und der Krankheitskontrollrate bei Patienten mit Melanom im Stadium IV, die mit einer PD-1-basierten Erstlinien-Immuntherapie behandelt wurden. Patienten mit vollständigem Ansprechen, teilweisem Ansprechen und stabilem Krankheitsverlauf als bestem Gesamtansprechen (BOR) gemäß RECIST 1.1 wurden in die Krankheitskontrollgruppe aufgenommen. Patienten mit fortschreitender Erkrankung als BOR wurden in die primäre Resistenzgruppe aufgenommen. Sekundäre Endpunkte waren PFS und OS.

Von den 319 Patienten erlitten 53 % mindestens eine irAE. Die Krankheit wurde bei einem höheren Prozentsatz der Patienten mit irAEs kontrolliert als bei Patienten ohne irAEs, unabhängig vom CTCAE-Grad (69,8 % gegenüber 49,3 %). In der multivariaten Analyse war die Entwicklung von irAEs der Grade 3 und 4 signifikant mit einem Schutzeffekt für das Ergebnis primäre Resistenz verbunden (OR: 0,40 95% CI 0,23-0,70, p=0,001). Das Vorhandensein von irAEs jeglichen Grades war signifikant mit einem längeren OS assoziiert (irAEs Grad 1-2 HRadj: 0,61 95% CI: 0,4-0,93, p=0,02, irAEs Grad 3-4 HRadj: 0,55 95% CI 0,31-0,99, p=0,04), jedoch nicht mit dem PFS (irAEs Grad 1-2 HRadj: 1,21 95% CI: 0,91-1,79, p=0,16, irAEs Grad 3-4 HRadj: 1,14 95% CI 0,83-2,02, p=0,24).

Schlussfolgerung: Das Vorhandensein von irAEs mit Laborexpression ist positiv mit dem Ansprechen und dem OS assoziiert, was darauf hindeutet, dass irAEs in dieser Situation tatsächlich ein prädiktiver Faktor sein könnten.

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8 DECLARATION OF CONTRIBUTION OF OTHERS

Statutory Declaration

Hereby I affirm that I wrote this Doctoral thesis independently with the topic **"Population based and clinical epidemiologic studies on different forms of skin cancer"** and that I used no other aids than those cited. In each individual case, I have clearly identified the source of the passages that are taken paraphrased from other works and the linked manuscript. The first study was carried out in the Institute of Clinical Epidemiology and Applied Biostatistics, University Hospital of Tübingen under the supervision of the Prof. Peter Martus, and in the Institute for Medical Virology and Epidemiology of Viral Diseases, University Hospital of Tübingen under the supervision of the Prof. Thomas Iftner. The second study was carried out in the Institute of Clinical Epidemiology and Applied Biostatistics, University Hospital of Tübingen under the supervision of the Prof. Peter Martus, and in the Skin Cancer Clinical Trials center of University Hospital of Tübingen under the supervision of the Prof. Dr. Thomas Kurt Eigentler and Dr. Teresa Amaral. The first study was designed by Prof. Dr. Thomas Iftner, Prof. Dr. Claus Garbe, Prof. Dr. Simone Harrison, Prof. Dr. Beverly Raasch, and Dr. Petra Buttner, together with myself. The second study was designed by Prof. Dr. Thomas Kurt Eigentler, Prof. Dr. Claus Garbe and Dr. Teresa Amaral, together with myself. The patients' evaluation and sample collection from the first study was performed by Dr. Simone Harrison, Prof. Dr. Beverly Raasch, Dr. Petra Buttner and Mrs. Margaret Glasby. The data collected from the second study was done by Prof. Dr. Thomas Kurt Eigentler, Prof. Dr. Ulrike Leiter, PD. Dr. Andrea Forschner, Dr. Olivia Seeber and Dr. Teresa Amaral. Dr Teresa Amaral provided also the data for the study number 2. The majority of the analysis were performed by me, following the advices of other colleagues, specifically Prof. Dr Peter Martus, Prof. Dr. Thomas Iftner, Prof. Dr. Claus Garbe, Prof. Dr. Ulrike Leiter-Stöppke and Dr. Teresa Amaral.

Prof. Dr. Thomas Iftner, Prof. Dr. Claus Garbe, Prof. Dr. Ulrike Leiter, Dr. Simone Harrison and Prof. Dr. Peter Martus contributed to the supervision of the analysis

performed and edited the first manuscript. Prof. Dr. Thomas Kurt Eigentler, Prof. Dr. Claus Garbe, Prof. Dr. Ulrike Leiter, Prof. Dr. Peter Martus and Dr. Teresa Amaral contributed to the supervision of the analysis performed and edited the second manuscript. I acknowledge the work done by collaborators. I affirm to have completed the manuscript independently and I performed the scientific studies according to the principles of good scientific practice.

I appreciate your attention to this matter and look forward to proceeding with the examination process. If any additional information or documentation is required, please do not hesitate to contact me at Lina.serna-higuita@med.uni-tuebingen.de or Phone Number: 017634396702

Thank you for your guidance and support throughout my doctoral journey.

Sincerely,

Lina Maria Serna Higuita